

CONSTRUCTION AND USE OF RECOMBINANT PARAINFLUENZA VIRUSES EXPRESSING A CHIMERIC GLYCOPROTEIN

RELATED APPLICATIONS

5 The present application is a continuation-in-part application of, and claims the
benefit under Title 35 of, U.S. Patent Application No. 09/083,793, filed May 22, 1998 which is
a continuation-in-part of U.S. Provisional Application No. 60/047,575, filed May 23, 1997,
now abandoned, and U.S. Provisional Application No. 60/059,385, filed September 19, 1997,
now abandoned. The disclosures of each of the foregoing priority applications are
10 incorporated herein by reference in their entirety for all purposes.

BACKGROUND OF THE INVENTION

Human parainfluenza virus type 3 (HPIV3) is a common cause of serious lower
respiratory tract infection in infants and children less than one year of age. It is second only to
15 respiratory syncytial virus (RSV) as a leading cause of hospitalization for viral lower
respiratory tract disease in this age group (Collins et al., p. 1205-1243. In B. N. Fields (Knipe
et al., eds), *Fields Virology*, 3rd ed, vol. 1. Lippincott-Raven Publishers, Philadelphia, 1996;
Crowe et al., *Vaccine* 13:415-421, 1995; Marx et al., *J. Infect. Dis.* 176:1423-1427, 1997).
Infections by this virus results in substantial morbidity in children less than 3 years of age.
20 HPIV1 and HPIV2 are the principal etiologic agents of laryngotracheobronchitis (croup) and
also can cause severe pneumonia and bronchiolitis (Collins et al., 3rd ed. In "*Fields Virology*,"
B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B.
Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1205-1243. Lippincott-Raven Publishers,
Philadelphia, 1996). In a long term study over a 20-year period, HPIV1, HPIV2, and HPIV3
25 were identified as etiologic agents for 6.0, 3.2, and 11.5%, respectively, of hospitalizations for
respiratory tract disease accounting in total for 18% of the hospitalizations, and, for this reason,
there is a need for an effective vaccine (Murphy et al., *Virus Res* 11, 1-15, 1988). The
parainfluenza viruses have also been identified in a significant proportion of cases of virally-
induced middle ear effusions in children with otitis media (Heikkinen et al., *N Engl J Med*
30 340:260-4, 1999). Thus, there is a need to produce a vaccine against these viruses that can
prevent the serious lower respiratory tract disease and the otitis media that accompanies these
HPIV infections. HPIV1, HPIV2, and HPIV3 are distinct serotypes which do not elicit

significant cross-protective immunity.

Despite considerable efforts to develop effective vaccine therapies against HPIV, no approved vaccine agents have yet been achieved for any HPIV serotype, nor for ameliorating HPIV related illnesses. To date, only two live attenuated PIV vaccine candidates have received particular attention. One of these candidates is a bovine PIV (BPIV3) strain that is antigenically related to HPIV3 and which has been shown to protect animals against HPIV3. BPIV3 is attenuated, genetically stable and immunogenic in human infants and children (Karron et al., J. Inf. Dis. 171:1107-14 (1995a); Karron et al., J. Inf. Dis. 172:1445-1450, (1995b)). A second PIV3 vaccine candidate, JS *cp45*, is a cold-adapted mutant of the JS wildtype (wt) strain of HPIV3 (Karron et al., (1995b), *supra*; Belshe et al., J. Med. Virol. 10:235-42 (1982)). This live, attenuated, cold-passaged (*cp*) PIV3 vaccine candidate exhibits temperature-sensitive (*ts*), cold-adaptation (*ca*), and attenuation (*att*) phenotypes which are stable after viral replication *in vivo*. The *cp45* virus is protective against human PIV3 challenge in experimental animals and is attenuated, genetically stable, and immunogenic in seronegative human infants and children (Hall et al., Virus Res. 22:173-184 (1992); Karron et al., (1995b), *supra*). The most promising prospects to date are live attenuated vaccine viruses since these have been shown to be efficacious in non-human primates even in the presence of passively transferred antibodies, an experimental situation that simulates that present in the very young infant who possesses maternally acquired antibodies (Crowe et al., Vaccine 13:847-855, 1995; Durbin et al., J Infect Dis 179:1345-1351, 1999). Two live attenuated PIV3 vaccine candidates, a temperature-sensitive (*ts*) derivative of the wild type PIV3 JS strain (designated PIV3*cp45*) and a bovine PIV3 (BPIV3) strain, are undergoing clinical evaluation (Karron et al., Pediatr Infect Dis J 15:650-654, 1996; Karron et al., J Infect Dis 171:1107-1114, 1995a; Karron et al., J Infect Dis 172, 1445-1450, 1995b). The live attenuated PIV3*cp45* vaccine candidate was derived from the JS strain of HPIV3 via serial passage in cell culture at low temperature and has been found to be protective against HPIV3 challenge in experimental animals and to be satisfactorily attenuated, genetically stable, and immunogenic in seronegative human infants and children (Belshe et al, J. Med. Virol. 10:235-242, 1982; Belshe et al., Infect Immun 37:160-5, 1982; Clements et al., J. Clin. Microbiol. 29:1175-82, 1991; Crookshanks et al., J. Med. Virol. 13:243-9, 1984; Hall et al., Virus Res. 22:173-184, 1992; Karron et al., J. Infect. Dis. 172, 1445-1450, 1995b). Because these PIV3 candidate vaccine viruses are biologically derived, there is no proven methods for adjusting the level of attenuation should this be found necessary from ongoing clinical trials.

To facilitate development of PIV vaccine candidates, recombinant DNA technology has recently made it possible to recover infectious negative-stranded RNA viruses from cDNA (for reviews, see Conzelmann, J. Gen. Virol. 77:381-89 (1996); Palese et al., Proc. Natl. Acad. Sci. U.S.A. 93:11354-58, (1996)). In this context, recombinant rescue has been reported for infectious respiratory syncytial virus (RSV), rabies virus (RaV), simian virus 5 (SV5), rinderpest virus, Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), measles virus (MeV), and Sendai virus (SeV) from cDNA-encoded antigenomic RNA in the presence of essential viral proteins (see, e.g., Garcin et al., EMBO J. 14:6087-6094 (1995); Lawson et al., Proc. Natl. Acad. Sci. U.S.A. 92:4477-81 (1995); Radecke et al., EMBO J. 14:5773-5784 (1995); Schnell et al., EMBO J. 13:4195-203 (1994); Whelan et al., Proc. Natl. Acad. Sci. U.S.A. 92:8388-92 (1995); Hoffman et al., J. Virol. 71:4272-4277 (1997); Kato et al., Genes to Cells 1:569-579 (1996), Roberts et al., Virology 247(1), 1-6 (1998); Baron et al., J. Virol. 71:1265-1271 (1997); International Publication No. WO 97/06270; Collins et al., Proc. Natl. Acad. Sci. USA 92:11563-11567 (1995); U.S. Patent Application No. 08/892,403, filed July 15, 1997 (corresponding to published International Application No. WO 98/02530 and priority U.S. Provisional Application Nos. 60/047,634, filed May 23, 1997, 60/046,141, filed May 9, 1997, and 60/021,773, filed July 15, 1996); U.S. Patent Application Serial No. 09/291,894, filed by Collins et al. on April 13, 1999; U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999; U.S. Provisional Patent Application Serial No. 60/143,132, filed by Bucholz et al. on July 9, 1999; Juhasz et al., J. Virol. 71(8):5814-5819 (1997); He et al. Virology 237:249-260 (1997); Peters et al. J. Virol. 73:5001-5009, 1999; Baron et al. J. Virol. 71:1265-1271 (1997); Whitehead et al., Virology 247(2):232-9 (1998a); Whitehead et al., J. Virol. 72(5):4467-4471 (1998b); Jin et al. Virology 251:206-214 (1998); Bucholz et al. J. Virol. 73:251-259 (1999); and Whitehead et al., J. Virol. 73:(4)3438-3442 (1999), each incorporated herein by reference in its entirety for all purposes).

In more specific regard to the instant invention, a method for producing HPIV with a wt phenotype from cDNA was recently developed for recovery of infectious, recombinant HPIV3 JS strain (see, e.g., Durbin et al., Virology 235:323-332, 1997; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997, each incorporated herein by reference). In addition, these disclosures allow for genetic manipulation of viral cDNA clones to determine the genetic basis of phenotypic changes in biological mutants, e.g., which mutations in the HPIV3 *cp45* virus specify its *ts*, *ca* and *att*

phenotypes, and which gene(s) or genome segment(s) of BPIV3 specify its attenuation phenotype. Additionally, these and related disclosures render it feasible to construct novel PIV vaccine candidates having a wide range of different mutations and to evaluate their level of attenuation, immunogenicity and phenotypic stability (see also, U.S. Provisional Patent Application Serial No. 60/143,134, filed by Bailey et al. on July 9, 1999; and U.S. Patent Application Serial No. 09/350,821, filed by Durbin et al. on July 9, 1999, each incorporated herein by reference).

Thus, infectious wild type recombinant PIV3, (r)PIV3, as well as a number of *ts* derivatives, have now been recovered from cDNA, and reverse genetics systems have been used to generate infectious virus bearing defined attenuating mutations and to study the genetic basis of attenuation of existing vaccine viruses. For example, the three amino acid substitutions found in the L gene of *cp45*, singularly or in combination, have been found to specify the *ts* and attenuation phenotypes. Additional *ts* and attenuating mutations are present in other regions of the PIV3*cp45*. In addition a chimeric PIV1 vaccine candidate has been generated using the PIV3 cDNA rescue system by replacing the PIV3 HN and F open reading frames (ORFs) with those of PIV1 in a PIV3 full-length cDNA that contains the three attenuating mutations in L. The recombinant chimeric virus derived from this cDNA is designated rPIV3-1.*cp45*L (Skiadopoulos et al., J Virol 72:1762-8, 1998; Tao et al., J Virol 72:2955-2961, 1998; Tao et al., Vaccine 17:1100-1108, 1999, incorporated herein by reference). rPIV3-1.*cp45*L was attenuated in hamsters and induced a high level of resistance to challenge with PIV1. Yet another recombinant chimeric virus, designated rPIV3-1.*cp45*, has been produced that contains 12 of the 15 *cp45* mutations, i.e., excluding the mutations that occur in HN and F. This recombinant vaccine candidate is highly attenuated in the upper and lower respiratory tract of hamsters and induces a high level of protection against HPIV1 infection (Skiadopoulos et al., Vaccine In press, 18:503-510, 1999).

Recently, a number of studies have focused on the possible use of viral vectors to express foreign antigens toward the goal of developing vaccines against a pathogen for which other vaccine alternatives are not proved successful. In this context, a number of reports suggest that foreign genes may be successfully inserted into a recombinant negative strand RNA virus genome or antigenome with varying effects (Bukreyev et al., J. Virol. 70:6634-41, 1996; Bukreyev et al., Proc. Natl. Acad. Sci. U S A 96:2367-72, 1999; Finke et al. J. Virol. 71:7281-8, 1997; Hasan et al., J. Gen. Virol. 78:2813-20, 1997; He et al., Virology 237:249-60, 1997; Jin et al., Virology 251:206-14, 1998; Johnson et al., J. Virol. 71:5060-8, 1997; Kahn et

al., Virology 254:81-91, 1999; Kretzschmar et al., J. Virol. 71:5982-9, 1997; Mebatsion et al., Proc. Natl. Acad. Sci. U S A 93:7310-4, 1996; Moriya et al., FEBS Lett. 425:105-11, 1998; Roberts et al., J. Virol. 73:3723-32, 1999; Roberts et al., J. Virol. 72:4704-11, 1998; Roberts et al., Virology 247:1-6, 1998; Sakai et al., FEBS Letter 456:221-226, 1999; Schnell et al., Proc. Natl. Acad. Sci. U S A 93:11359-65, 1996a; Schnell et al., J. Virol. 70:2318-23, 1996b; Schnell et al., Cell 90:849-57, 1997; Singh et al., J. Gen. Virol. 80:101-6, 1999; Singh et al., J. Virol. 73:4823-8, 1999; Spielhofer et al., J. Virol. 72, 2150-9, 1998; Yu et al., Genes to Cells 2:457-66 et al., 1999; U.S. Provisional Patent Application Serial No. 60/143,425, filed on July 13, 1999, each incorporated herein by reference). When inserted into the viral genome under the control of viral transcription gene-start and gene-end signals, the foreign gene may be transcribed as a separate mRNA and yield significant protein expression. Surprisingly, in some cases foreign sequence has been reported to be stable and capable of expressing functional protein during numerous passages *in vitro*.

However, to successfully develop vectors for vaccine use, it is insufficient to simply demonstrate a high, stable level of protein expression. For example, this has been possible since the early-to-mid 1980s with recombinant vaccinia viruses and adenoviruses, and yet these vectors have proven to be disappointments in the development of vaccines for human use. Similarly, most nonsegmented negative strand viruses which have been developed as vectors do not possess properties or immunization strategies amenable for human use. Examples in this context include vesicular stomatitis virus, an ungulate pathogen with no history of administration to humans except for a few laboratory accidents; Sendai virus, a mouse pathogen with no history of administration to humans; simian virus 5, a canine pathogen with no history of administration to humans; and an attenuated strain of measles virus which must be administered systemically and would be neutralized by measles-specific antibodies present in nearly all humans due to maternal antibodies and widespread use of a licensed vaccine. Furthermore, some of these prior vector candidates have adverse effects, such as immunosuppression, which are directly inconsistent with their use as vectors. Thus, one must identify vectors whose growth characteristics, tropisms, and other biological properties make them appropriate as vectors for human use. It is further necessary to develop a viable vaccination strategy, including an immunogenic and efficacious route of administration.

Among a host of human pathogens for which a vector-based vaccine approach may be desirable is the measles virus. A live attenuated vaccine has been available for more than three decades and has been largely successful in eradicating measles disease in the United

States. However, the World Health Organization estimates that more than 45 million cases of measles still occur annually, particularly in developing countries, and the virus contributes to approximately one million deaths per year

Measles virus is a member of the *Morbillivirus* genus of the *Paramyxoviridae* family (Griffin et al., In "Fields Virology", B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996). It is one of the most contagious infectious agents known to man and is transmitted from person to person via the respiratory route (Griffin et al., In "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds.), Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996). The measles virus has a complex pathogenesis, involving replication in both the respiratory tract and various systemic sites (Griffin et al., In "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds.), Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996).

Although both mucosal IgA and serum IgG measles virus-specific antibodies can participate in the control of measles virus, the absence of measles virus disease in very young infants possessing maternally-acquired measles virus-specific antibodies identifies serum antibodies as a major mediator of resistance to disease (Griffin et al., In "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds.), Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996). The two measles virus glycoproteins, the hemagglutinin (HA) and fusion (F) proteins, are the major neutralization and protective antigens (Griffin et al., In "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds.), Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996).

The currently available live attenuated measles vaccine is administered by a parenteral route (Griffin et al., In "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds.), Vol. 1, pp. 1267-1312. Lippincott-Raven Publishers, Philadelphia, 1996). Both the wild type measles virus and the vaccine virus are very readily neutralized by antibodies, and the measles virus vaccine is rendered non-infectious by even very low levels of maternally-acquired measles

virus-specific neutralizing antibodies (Halsey et al., N. Engl. J. Med. 313:544-9, 1985; Osterhaus et al., Vaccine 16:1479-81, 1998). Thus, the vaccine virus is not given until the passively-acquired maternal antibodies have decreased to undetectable levels. In the United States, measles virus vaccine is not given until 12 to 15 months of age, a time when almost all children are readily infected with the measles virus vaccine. In the developing world, measles virus continues to have a high mortality rate, especially in children within the latter half of the first year of life (Gellin et al., J. Infect. Dis. 170, S3-14, 1994; Taylor et al., Am. J. Epidemiol. 127:788-94, 1988). This occurs because the measles virus, which is highly prevalent in these regions, is able to infect that subset of infants in whom maternally-acquired measles virus-specific antibody levels have decreased to a non-protective level. Therefore, there is a need for a measles virus vaccine that is able to induce a protective immune response even in the presence of measles virus neutralizing antibodies with the goal of eliminating measles virus disease occurring within the first year of life as well as that which occurs thereafter. Given this need, there have been numerous attempts to develop an immunization strategy to protect infants in the latter half of the first year of life against measles virus, but none of these strategies has been effective to date.

The first strategy for developing an early measles vaccine involved administration of the licensed live attenuated measles virus vaccine to infants about six months of age by one of the following two methods (Cutts et al., Biologicals 25, 323-38, 1997). In one general protocol, the live attenuated measles virus was administered intranasally by drops (Black et al., New Eng. J. Med. 263, 165-169; 1960; Kok et al., Trans. R. Soc. Trop. Med. Hyg. 77:171-6, 1983; Simasathien et al., Vaccine 15:329-34, 1997) or into the lower respiratory tract by aerosol (Sabin et al., J. Infect. Dis. 152:1231-7, 1985), to initiate an infection of the respiratory tract. In a second protocol, the measles virus was given parenterally but at a higher dose than that employed for the current vaccine. The administration of vaccines that can replicate on mucosal surfaces has been successfully achieved in early infancy for both live attenuated poliovirus and rotavirus vaccines (Melnick et al., In "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds.), Vol. 1, pp. 655-712. 2 vols. Lippencott-Raven Publishers, Philadelphia, 1996; Perez-Schael et al., N. Engl. J. Med. 337, 1181-7, 1997), presumably because passively-acquired IgG antibodies have less access to mucosal surfaces than they do to systemic sites of viral replication. In this situation, the live attenuated poliovirus vaccine viruses are able to infect the mucosal surface of the

gastrointestinal tract or the respiratory tract of young infants, including those with maternal antibodies, resulting in the induction of a protective immune response.

Therefore, a plausible method is to immunize via the respiratory tract of the young infant with the live attenuated measles virus vaccine, since this is the natural route of infection with the measles virus. However, the live attenuated measles virus that is infectious by the parenteral route was inconsistently infectious by the intranasal route (Black et al., New Eng. J. Med. 263:165-169, 1960; Cutts et al., Biologicals 25, 323-38, 1997; Kok et al., Trans. R. Soc. Trop. Med. Hyg. 77:171-6, 1983; Simasathien et al., Vaccine 15:329-34, 1997), and this decreased infectivity was especially apparent for the Schwartz strain of measles virus vaccine which is the current vaccine strain. Presumably, during the attenuation of this virus by passage in tissue culture cells of avian origin, the virus lost a significant amount of infectivity for the upper respiratory tract of humans. Indeed, a hallmark of measles virus biology is that the virus undergoes rapid changes in biological properties when grown *in vitro*. Since this relatively simple route of immunization was not successful, a second approach was tried involving administration of the live virus vaccine by aerosol into the lower respiratory tract (Cutts et al., Biologicals 25, 323-38, 1997; Sabin et al., J. Infect. Dis. 152:1231-7, 1985).

Infection of young infants by aerosol administration of measles virus vaccine was accomplished in highly controlled experimental studies, but it has not been possible to reproducibly deliver a live attenuated measles virus vaccine in field settings by aerosol to the young uncooperative infant (Cutts et al., Biologicals 25, 323-38, 1997). In another attempt to immunize six-month old infants, the measles vaccine virus was administered parenterally at a 10- to 100-fold increased dose (Markowitz et al., N. Engl. J. Med. 322:580-7, 1990). Although high-titer live measles vaccination improved seroconversion in infants 4-6 months of age, there was an associated increase in mortality in the high-titer vaccine recipients later in infancy (Gellin et al., J. Infect. Dis. 170:S3-14, 1994; Holt et al., J. Infect. Dis. 168:1087-96, 1993; Markowitz et al., N. Engl. J. Med. 322:580-7, 1990) and this approach to immunization has been abandoned.

A second strategy previously explored for a measles virus vaccine was the use of an inactivated measles virus vaccine, specifically, a formalin inactivated whole measles virus or a subunit virus vaccine prepared from measles virus (Griffin et al., In "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds.), Vol. 1, pp. 1267-1312. Lippincott-Raven

Publishers, Philadelphia, 1996). However, the clinical use of the vaccines in the 1960's revealed a very serious complication, namely, that the inactivated virus vaccines potentiated disease rather than prevented it (Fulginiti et al., JAMA 202:1075-80, 1967). This was first observed with formalin-inactivated measles virus vaccine (Fulginiti et al., JAMA 202:1075-80, 1967). Initially, this vaccine prevented measles, but after several years vaccinees lost their resistance to infection. When subsequently infected with naturally circulating measles virus, the vaccinees developed an atypical illness with accentuated systemic symptoms and pneumonia (Fulginiti et al., JAMA 202:1075-80, 1967; Nader et al., J. Pediatr. 72:22-8, 1968; Rauh et al., Am. J. Dis. Child 109:232-7, 1965). Retrospective analysis showed that formalin inactivation destroyed the ability of the measles fusion (F) protein to induce hemolysis-inhibiting antibodies, but it did not destroy the ability of the HA (hemagglutinin or attachment) protein to induce neutralizing antibodies (Norrby et al., J. Infect. Dis. 132:262-9, 1975; Norrby et al., Infect. Immun. 11:231-9, 1975). When the immunity induced by the HA protein had waned sufficiently to permit extensive infection with wild type measles virus, an altered and sometimes more severe disease was seen at the sites of measles virus replication (Bellanti, Pediatrics 48:715-29, 1971; Buser, N. Engl. J. Med. 277:250-1, 1967). This atypical disease is believed to be mediated in part by an altered cell-mediated immune response in which Th-2 cells were preferentially induced leading to heightened disease manifestations at the sites of viral replication (Polack et al., Nat. Med. 5:629-34, 1999). Because of this experience with nonliving measles virus vaccines and also because the immunogenicity of such parenterally-administered vaccines can be decreased by passively-transferred antibodies, there has been considerable reluctance to evaluate such vaccines in human infants. It should be noted that disease potentiation appears to be associated only with killed vaccines.

Yet another strategy that has been explored for developing a vaccine against measles for use in young infants has been the use of viral vectors to express a protective antigen of the measles virus (Drillien et al., Proc. Natl. Acad. Sci. U S A 85:1252-6, 1988; Fooks et al., J. Gen. Virol. 79:1027-31, 1998; Schnell et al., Proc. Natl. Acad. Sci. U S A 93:11359-65, 1996a; Taylor et al., Virology 187:321-8, 1992; Wild et al., Vaccine 8:441-2, 1990; Wild et al., J. Gen. Virol. 73:359-67, 1992). A variety of vectors have been explored including poxviruses such as the replication-competent vaccinia virus or the replication-defective modified vaccinia virus Ankara (MVA) strain. Replication-competent vaccinia recombinants expressing the F or HA glycoprotein of measles virus were efficacious in immunologically naive vaccinees. However, when they were administered parenterally in the presence of passive antibody against measles virus, their immunogenicity and protective

efficacy was largely abrogated (Galletti et al., Vaccine 13, 197-201, 1995; Osterhaus et al., Vaccine 16:1479-81, 1998; Siegrist et al., Vaccine 16:1409-14, 1998; Siegrist et al., Dev. Biol. Stand. 95:133-9, 1998).

Replication-competent vaccinia recombinants expressing the protective antigens of RSV have also been shown to be ineffective in inducing a protective immune response when they are administered parenterally in the presence of passive antibody (Murphy et al., J. Virol. 62:3907-10, 1988a), but they readily protected such hosts when administered intranasally. Unfortunately, replication-competent vaccinia virus recombinants are not sufficiently attenuated for use in immunocompromised hosts such as persons with human immunodeficiency virus (HIV) infection (Fenner et al., World Health Organization, Geneva, 1988; Redfield et al., N. Engl. J. Med. 316, 673-676, 1987), and their administration by the intranasal route even to immunocompetent individuals would be problematic. Therefore they are not being pursued as vectors for use in human infants, some of whom could be infected with HIV.

The MVA vector, which was derived by more than 500 passages in chick embryo cells (Mayr et al., Infection 3:6-14, 1975; Meyer et al., J. Gen. Virol. 72:1031-1038, 1991), has also been evaluated as a potential vaccine vector for the protective antigens of several paramyxoviruses (Durbin et al., J. Infect. Dis. 179:1345-51, 1999a; Wyatt et al., Vaccine 14, 1451-1458, 1996). MVA is a highly attenuated host range mutant that replicates well in avian cells but not in most mammalian cells, including those obtained from monkeys and humans (Blanchard et al., J. Gen. Virol. 79:1159-1167, 1998; Carroll et al., Virology 238:198-211, 1997; Drexler et al., J. Gen. Virol. 79, 347-352, 1998; Sutter et al., Proc. Natl. Acad. Sci. U.S. A. 89:10847-10851, 1992). Avipox vaccine vectors, which have a host range restriction similar to that of MVA, also have been constructed that express measles virus protective antigens (Taylor et al., Virology 187, 321-8, 1992). MVA is non-pathogenic in immunocompromised hosts and has been administered to large numbers of humans without incident (Mayr et al., Zentralbl Bakteriologie [B] 167, 375-90, 1978; Stickl et al., Dtsch. Med. Wochenschr. 99:2386-92, 1974; Werner et al., Archives of Virology 64, 247-256, 1980). Unfortunately, both the immunogenicity and efficacy of MVA expressing a paramyxovirus protective antigen were abrogated in passively-immunized rhesus monkeys whether delivered by a parenteral or a topical route (Durbin et al., Virology 235:323-332, 1999). The immunogenicity of DNA vaccines expressing measles virus protective antigens delivered parenterally was also decreased in passively-immunized hosts (Siegrist et al., Dev. Biol. Stand.

95:133-9, 1998). Replication-defective vectors expressing measles virus protective antigens are presently being evaluated, including adenovirus-measles virus HA recombinants (Fooks et al., J. Gen. Virol. 79:1027-31, 1998). In this context, MVA recombinants expressing parainfluenza virus antigens, unlike replication-competent vaccinia virus recombinants, lacked protective efficacy when given by a mucosal route to animals with passively-acquired antibodies, and it is unlikely that they, or the similar avipox vectors, can be used in infants with maternally-acquired measles virus antibodies.

Based on the reports summarized above, it appears unlikely that a replication-competent or replication-defective poxvirus vector, or a DNA vaccine, expressing a measles virus protective antigen will be satisfactorily immunogenic or efficacious in infants possessing passively-acquired maternal measles virus-specific antibodies.

A recently developed replication-competent virus vector expressing measles virus HA that replicates in the respiratory tract of animal hosts has been developed, namely, vesicular stomatitis virus (VSV), a rhabdovirus which naturally infects cattle but not humans (Roberts et al., J. Virol. 73:3723-32, 1999; Schnell et al., Proc. Natl. Acad. Sci. U S A 93:11359-65, 1996a). Since VSV is an animal virus that can cause disease in humans, development of this recombinant for use in humans will require that a VSV backbone that is satisfactorily attenuated in human infants be first identified (Roberts et al., J. Virol. 73:3723-32, 1999), but such clinical studies have not been initiated.

Although there have been numerous advances toward development of effective vaccine agents against PIV and other pathogens, including measles, there remains a clear need in the art for additional tools and methods to engineer safe and effective vaccines to alleviate the serious health problems attributable to these pathogens, particularly among young infants. Among the remaining challenges in this context is the need for additional tools to generate suitably attenuated, immunogenic and genetically stable vaccine candidates for use in diverse clinical settings against one or more pathogens. To facilitate these goals, existing methods for identifying and incorporating attenuating mutations into recombinant vaccine strains and for developing vector-based vaccines and immunization methods must be expanded. Surprisingly, the present invention fulfills these needs and provides additional advantages as described herein below.

SUMMARY OF THE INVENTION

The present invention provides chimeric parainfluenza viruses (PIVs) that are infectious in humans and other mammals and are useful in various compositions to generate desired immune responses against one or more PIVs, or against a PIV and one or more additional pathogens in a host susceptible to infection therefrom. In preferred aspects, the invention provides novel methods for designing and producing attenuated, chimeric PIVs that are useful as vaccine agents for preventing and/or treating infection and related disease symptoms attributable to PIV and one or more additional pathogens. Included within these aspects of the invention are novel, isolated polynucleotide molecules and vectors incorporating such molecules that comprise a chimeric PIV genome or antigenome including a partial or complete PIV vector genome or antigenome combined or integrated with one or more heterologous genes or genome segments that encode single or multiple antigenic determinants of a heterologous pathogen or of multiple heterologous pathogens. Also provided within the invention are methods and compositions incorporating a chimeric PIV for prophylaxis and treatment of infection by both a selected PIV and one or more heterologous pathogens, e.g., a heterologous PIV or a non-PIV pathogen such as a measles virus.

The invention thus involves methods and compositions for developing live vaccine candidates based on chimeras that employ a parainfluenza virus or subviral particle that is recombinantly modified to incorporate one or more antigenic determinants of a heterologous pathogen(s). Chimeric PIVs of the invention are constructed through a cDNA-based virus recovery system. Recombinant chimeric PIVs made from cDNA replicate independently and are propagated in a similar manner as biologically-derived viruses. The recombinant viruses are engineered to incorporate nucleotide sequences from both a vector (i.e., a "recipient" or "background") PIV genome or antigenome, and one or more heterologous "donor" sequences encoding one or more antigenic determinants of a different PIV or heterologous pathogen—to produce an infectious, chimeric virus or subviral particle. In this manner, candidate vaccine viruses are recombinantly engineered to elicit an immune response against one or more PIVs or a polyspecific response against a selected PIV and a non-PIV pathogen in a mammalian host susceptible to infection therefrom. Preferably the PIV and/or non-PIV pathogen(s) from which the heterologous sequences encoding the antigenic determinant(s) are human pathogens and the host is a human host. Also preferably, the vector PIV is a human PIV, although non-human PIVs, for example a bovine PIV (BPIV), can be employed as a vector to incorporate antigenic determinants of human PIVs and other human

pathogens. Chimeric PIVs according to the invention may elicit an immune response against a specific PIV, e.g., HPIV1, HPIV2, HPIV3, or a polyspecific immune response against multiple PIVs, e.g., HPIV1 and HPIV2. Alternatively, chimeric PIVs of the invention may elicit a polyspecific immune response against one or more PIVs and a non-PIV pathogen such as measles virus.

Exemplary chimeric PIV of the invention incorporate a chimeric PIV genome or antigenome as described above, as well as a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), and a large polymerase protein (L). Additional PIV proteins may be included in various combinations to provide a range of infectious subviral particles, up to a complete viral particle or a viral particle containing supernumerary proteins, antigenic determinants or other additional components.

Chimeric PIV of the invention include a partial or complete "vector" PIV genome or antigenome derived from or patterned after a human PIV or non-human PIV combined with one or more heterologous gene(s) or genome segment(s) of a different PIV or other pathogen to form the chimeric PIV genome or antigenome. In preferred aspects of the invention, chimeric PIV incorporate a partial or complete human PIV vector genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a second human PIV or a non-PIV pathogen such as measles virus.

The PIV "vector" genome or antigenome typically acts as a recipient or carrier to which are added or incorporated one or more "donor" genes or genome segments of a heterologous pathogen. Typically, polynucleotides encoding one or more antigenic determinants of the heterologous pathogen are added to or substituted within the vector genome or antigenome to yield a chimeric PIV that thus acquires the ability to elicit an immune response in a selected host against the heterologous pathogen. In addition, the chimeric virus may exhibit other novel phenotypic characteristics compared to one or both of the vector PIV and heterologous pathogens. For example, addition or substitution of heterologous genes or genome segments within a vector PIV strain may additionally, or independently, result in an increase in attenuation, growth changes, or other desired phenotypic changes as compared with a corresponding phenotype of the unmodified vector virus and/or donor. In one aspect of the invention, chimeric PIVs are attenuated for greater efficacy as a vaccine candidate by incorporation of large polynucleotide inserts which specify the level of attenuation in the resulting chimeric virus dependent upon the size of the insert.

Preferred chimeric PIV vaccine candidates of the invention bear one or more major antigenic determinants of a human PIV, e.g., of HPIV1, HPIV2 or HPIV3, and thus elicit an effective immune response against the selected PIV in human hosts. The antigenic determinant which is specific for a selected human PIV may be encoded by the vector genome or antigenome, or may be inserted within or joined to the PIV vector genome or antigenome as a heterologous polynucleotide sequence from a different PIV. The major protective antigens of human PIVs are their HN and F glycoproteins, although other proteins can also contribute to a protective or therapeutic immune response. In this context, both humoral and cell mediated immune responses are advantageously elicited by representative vaccine candidates within the invention. Thus, polynucleotides encoding antigenic determinants that may be present in the vector genome or antigenome, or integrated therewith as a heterologous gene or genome segment, may encode one or more PIV N, P, C, D, V, M, F, HN and/or L protein(s) or selected immunogenic fragment(s) or epitope(s) thereof from any human PIV.

In addition to having one or more major antigenic determinants of a selected human PIV, preferred chimeric PIV vaccine viruses of the invention bear one or more major antigenic determinants of a second human PIV or of a non-PIV pathogen. In exemplary aspects, the chimeric PIV includes a vector genome or antigenome that is a partial or complete human PIV (HPIV) genome or antigenome, for example of HPIV3, and further includes one or more heterologous gene(s) or genome segment(s) encoding antigenic determinant(s) of at least one heterologous PIV, for example HPIV1 and/or HPIV2. Preferably, the vector genome or antigenome is a partial or complete HPIV3 genome or antigenome and the heterologous gene(s) or genome segment(s) encoding the antigenic determinant(s) is/are of one or more heterologous HPIV(s). In alternative embodiments, one or more genes or genome segments encoding one or more antigenic determinants of HPIV1 may be added to or substituted within the partial or complete HPIV3 genome or antigenome. Preferably, the antigenic determinant(s) of HPIV1 is/are selected from HPIV1 HN and F glycoproteins or comprise one or more antigenic domains, fragments or epitopes of the HN and/or F glycoproteins. In various exemplary embodiments, both of the HPIV1 genes encoding the HN and F glycoproteins are substituted for counterpart HPIV3 HN and F genes in the HPIV3 vector genome or antigenome. These constructs yield chimeric PIVs that elicit a mono- or poly-specific immune response in humans to HPIV3 and/or HPIV1.

In additional exemplary embodiments, one or more genes or genome segments encoding one or more antigenic determinants of HPIV2 is/are added to, or incorporated within,

a partial or complete HPIV3 genome or antigenome, yielding a new or additional immunospecificity of the resultant chimera against HPIV2 alone, or against HPIV3 and HPIV2. In more detailed aspects, one or more HPIV2 genes or genome segments encoding one or more HN and/or F glycoproteins or antigenic domains, fragments or epitopes thereof is/are added to or incorporated within the partial or complete HPIV3 vector genome or antigenome.

In yet additional aspects of the invention, multiple heterologous genes or genome segments encoding antigenic determinants of multiple heterologous PIVs are added to or incorporated within a partial or complete PIV vector genome or antigenome, preferably an HPIV vector genome or antigenome. In one preferred embodiment, heterologous genes or genome segments encoding antigenic determinants from both HPIV1 and HPIV2 are added to or incorporated within a partial or complete HPIV3 vector genome or antigenome. In more detailed aspects, one or more HPIV1 genes or genome segments encoding one or more HN and/or F glycoproteins (or antigenic domains, fragments or epitopes thereof) and one or more HPIV2 genes or genome segments encoding HN and/or F glycoproteins, antigenic domains, fragments or epitopes, is/are added to or incorporated within the partial or complete HPIV3 vector genome or antigenome. In one example, both HPIV1 genes encoding HN and F glycoproteins are substituted for counterpart HPIV3 HN and F genes to form a chimeric HPIV3-1 vector genome or antigenome, which is further modified by addition or incorporation of one or more genes or gene segments encoding single or multiple antigenic determinants of HPIV2. This is readily achieved within the invention, for example, by adding or substituting a transcription unit comprising an open reading frame (ORF) of an HPIV2 HN within the chimeric HPIV3-1 vector genome or antigenome. Following this method, specific constructs exemplifying the invention are provided which yield chimeric PIVs having antigenic determinants of both HPIV1 and HPIV2, as exemplified by the vaccine candidates rPIV3-1.2HN and rPIV3-1cp45.2HN described herein below.

In alternative aspects of the invention, chimeric PIVs of the invention are based on a human PIV vector genome or antigenome which is employed as a recipient for incorporation of major antigenic determinants from a non-PIV pathogen. Pathogens from which one or more antigenic determinants may be adopted into the chimeric PIV vaccine candidate include, but are not limited to, measles virus, subgroup A and subgroup B respiratory syncytial viruses, mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr

virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses. This assemblage of pathogens that may be thus targeted for vaccine development according to the methods of the invention is exemplary only, and those skilled in the art will understand that the use of PIV vectors for carrying antigenic determinants extends broadly to a large host of additional pathogens.

This, in various alternative aspects of the invention, a human PIV genome or antigenome can be employed as a vector for incorporation of one or more major antigenic determinants from a wide range of non-PIV pathogens. Representative major antigens that can be incorporated within chimeric PIVs of the invention include, but are not limited to the measles virus HA and F proteins; the F, G, SH and M2 proteins of subgroup A and subgroup B respiratory syncytial virus, mumps virus HN and F proteins, human papilloma virus L1 protein, type 1 or type 2 human immunodeficiency virus gp160 protein, herpes simplex virus and cytomegalovirus gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM proteins, rabies virus G Protein, Epstein Barr Virus gp350 protein; filovirus G protein, bunyavirus G protein, flavivirus E and NS1 proteins, and alphavirus E protein.

Various human PIV vectors can be employed to carry heterologous antigenic determinants of non-PIV pathogens to elicit one or more specific humoral or cell mediated immune responses against the antigenic determinant(s) carried by the chimeric vaccine virus and hence elicit an effective immune response against the wild-type "donor" pathogen in susceptible hosts. In preferred embodiments, one or more heterologous genes or genome segments from the donor pathogen is joined to or inserted within a partial or complete HPIV3 genome or antigenome. Alternatively, the heterologous gene or genome segment may be incorporated within a chimeric HPIV vector genome or antigenome, for example a partial or complete HPIV3 genome or antigenome bearing one or more genes or genome segments of a heterologous PIV. For example, the gene(s) or genome segment(s) encoding the antigenic determinant(s) of a non-PIV pathogen may be combined with a partial or complete chimeric HPIV3-1 vector genome or antigenome, e.g., as described above having one or both HPIV1 genes encoding HN and F glycoproteins substituted for counterpart HPIV3 HN and F genes. Alternatively, the gene(s) or genome segment(s) encoding the antigenic determinant(s) of a non-PIV pathogen may be combined with a partial or complete chimeric genome or antigenome that incorporates single or multiple antigenic determinants of HPIV2, e.g., an HPIV2 HN gene, within an HPIV1 or HPIV3 vector genome or antigenome, or a chimeric HPIV3-1 vector genome or antigenome as described above. The heterologous gene(s) or

genome segment(s) encoding one or more measles antigenic determinant(s) may be combined with any of the PIV vectors or chimeric PIV vectors disclosed herein. In the examples provided herein, the vector genome or antigenome is a partial or complete HPIV3 genome or antigenome, or a chimeric HPIV genome or antigenome comprising a partial or complete HPIV3 genome or antigenome having one or more genes or genome segments encoding antigenic determinant(s) of a heterologous HPIV added or incorporated therein. In one such chimeric construct, a transcription unit comprising an open reading frame (ORF) of a measles virus HA gene is added to a HPIV3 vector genome or antigenome at various positions, yielding exemplary chimeric PIV/measles vaccine candidates rPIV3(HA HN-L), rPIV3(HA N-P), rcp45L(HA N-P), rPIV3(HA P-M), or rcp45L(HA P-M).

To construct chimeric PIV clones of the invention, a heterologous gene or genome segment of a donor PIV or non-PIV pathogen may be added or substituted at any operable position in the vector genome or antigenome. Often, the position of a gene or gene segment substitution will correspond to a wild-type gene order position of a counterpart gene or genome segment within the partial or complete PIV vector genome or antigenome. In other embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promoter-distal compared to a wild-type gene order position of a counterpart gene or genome segment within the background genome or antigenome, to enhance or reduce expression, respectively, of the heterologous gene or genome segment.

In preferred detailed aspects of the invention, a heterologous genome segment, for example a genome segment encoding an immunogenic ectodomain of a heterologous PIV or non-PIV pathogen, can be substituted for a corresponding genome segment in a counterpart gene in the PIV vector genome or antigenome to yield constructs encoding chimeric proteins, e.g. fusion proteins having a cytoplasmic tail and/or transmembrane domain of one PIV fused to an ectodomain of another PIV or non-PIV pathogen. In alternate embodiments, a chimeric PIV genome or antigenome may be engineered to encode a polyspecific chimeric glycoprotein in the recombinant virus or subviral particle having immunogenic glycoprotein domains or epitopes from two different pathogens. In yet additional embodiments, heterologous genes or genome segments from one PIV or non-PIV pathogen can be added (i.e., without substitution) within a PIV vector genome or antigenome to create novel immunogenic properties within the resultant clone. In these cases, the heterologous gene or genome segment may be added as a supernumerary gene or genome segment, optionally for the additional purpose of attenuating the resultant chimeric virus, in combination with a complete PIV vector genome or

antigenome. Alternatively, the heterologous gene or genome segment may be added in conjunction with deletion of a selected gene or genome segment in the vector genome or antigenome.

In preferred embodiments of the invention, the heterologous gene or genome segment is added at an intergenic position within the partial or complete PIV vector genome or antigenome. Alternatively, the gene or genome segment can be inserted within other noncoding regions of the genome, for example, within 5' or 3' noncoding regions or in other positions where noncoding nucleotides occur within the vector genome or antigenome. In some instances, it may be desired to insert the heterologous gene or genome segment at a non-coding site corresponding to or overlapping a cis-acting regulatory sequence within the vector genome or antigenome, e.g., within a sequence required for efficient replication, transcription, and/or translation. These regions of the vector genome or antigenome represent target sites for disruption or modification of regulatory functions associated with introduction of the heterologous gene or genome segment.

For the preferred purpose of constructing candidate vaccine viruses for clinical use, it is often desirable to adjust the attenuation phenotype of chimeric PIV of the invention by introducing additional mutations that increase or decrease the level of attenuation in the recombinant virus. Therefore, in additional aspects of the invention, attenuated, chimeric PIVs are produced in which the chimeric genome or antigenome is further modified by introducing one or more attenuating mutations that specify an attenuating phenotype in the resultant virus or subviral particle. These attenuating mutations may be generated *de novo* and tested for attenuating effects according to well known rational design mutagenesis strategies. Alternatively, the attenuating mutations may be identified in existing biologically derived mutant PIV or other viruses and thereafter incorporated into a chimeric PIV of the invention.

Preferred attenuating mutations in the latter context are readily identified and incorporated into a chimeric PIV, either by inserting the mutation within the vector genome or antigenome by cloning or mutagenizing the vector genome or antigenome to contain the attenuating mutation. Preferably, attenuating mutations are engineered within the vector genome or antigenome and are imported or copied from biologically derived, attenuated PIV mutants. These are recognized to include, for example, cold passaged (*cp*), cold adapted (*ca*), host range restricted (*hr*), small plaque (*sp*), and/or temperature sensitive (*ts*) PIV mutants. In exemplary embodiments, one or more attenuating mutations present in the well characterized

JS HPIV3 *cp45* mutant strain are incorporated within chimeric PIV of the invention, preferably including one or more mutations identified in the polymerase L protein, e.g., at a position corresponding to Tyr₉₄₂, Leu₉₉₂, or Thr₁₅₅₈ of JS *cp45*. Alternatively or additionally, attenuating mutations present in the JS HPIV3 *cp45* mutant strain are introduced in the N protein of chimeric PIV clones, for example which encode amino acid substitution(s) at a position corresponding to residues Val₉₆ or Ser₃₈₉ of JS *cp45*. Yet additional useful attenuating mutations encode amino acid substitution(s) in the C protein, e.g., at a position corresponding to Ile₉₆ of JS *cp45*. Other mutations identified in PIV3 JS *cp45* that can be adopted to adjust attenuation of a chimeric PIV of the invention are found in the F protein, e.g., at a position corresponding to Ile₄₂₀ or Ala₄₅₀ of JS *cp45*, and in the HN protein, e.g., at a position corresponding to residue Val₃₈₄ of JS *cp45*.

Attenuating mutations from biologically derived PIV mutants for incorporation into chimeric PIV of the invention also include mutations in noncoding portions of the PIV genome or antigenome, for example in a 3' leader sequence. Exemplary mutations in this context may be engineered at a position in the 3' leader of a recombinant virus at a position corresponding to nucleotide 23, 24, 28, or 45 of JS *cp45*. Yet additional exemplary mutations may be engineered in the N gene start sequence, for example by changing one or more nucleotides in the N gene start sequence, e.g., at a position corresponding to nucleotide 62 of JS *cp45*.

From PIV3 JS *cp45* and other biologically derived PIV mutants, a large "menu" of attenuating mutations is provided, each of which mutations can be combined with any other mutation(s) for finely adjusting the level of attenuation in chimeric PIV vaccine candidates of the invention. In exemplary embodiments, chimeric PIVs are constructed which include one or more, and preferably two or more, mutations of HPIV3 JS *cp45*. Thus, chimeric PIVs of the invention selected for vaccine use often have two and sometimes three or more attenuating mutations from biologically derived PIV mutants or like model sources to achieve a satisfactory level of attenuation for broad clinical use. Preferably, these attenuating mutations incorporated within recombinant chimeric PIVs of the invention are stabilized by multiple nucleotide substitutions in a codon specifying the mutation.

Additional attenuating mutations can be readily adopted or engineered within chimeric PIVs of the invention that are identified in other viruses, particularly other nonsegmented negative stranded RNA viruses. This is accomplished by mapping a mutation

identified in a heterologous negative stranded RNA virus to a corresponding, homologous site in a PIV vector genome or antigenome (or heterologous insert in the PIV chimera) and mutating the existing sequence in the "recipient" to the mutant genotype (either by an identical or conservative mutation), as described in U.S. Provisional Patent Application Serial No.

5 60/129,006, filed on April 13, 1999, incorporated herein by reference.

In yet additional aspects of the invention, chimeric PIVs, with or without attenuating mutations modeled after biologically derived attenuated mutant viruses, are constructed to have additional nucleotide modification(s) to yield a desired phenotypic, structural, or functional change. Typically, the selected nucleotide modification will be made
10 within the partial or complete PIV vector genome, but such modifications can be made as well within any heterologous gene or genome segment that contributes to the chimeric clone. These modifications preferably specify a desired phenotypic change, for example a change in growth characteristics, attenuation, temperature-sensitivity, cold-adaptation, plaque size, host range restriction, or immunogenicity. Structural changes in this context include introduction or
15 ablation of restriction sites into PIV encoding cDNAs for ease of manipulation and identification.

In preferred embodiments, nucleotide changes within the genome or antigenome of a chimeric PIV include modification of a viral gene by partial or complete deletion of the gene or reduction or ablation (knock-out) of its expression. Target genes for mutation in this
20 context include any of the PIV genes, including the nucleocapsid protein N, phosphoprotein P, large polymerase subunit L, matrix protein M, hemagglutinin-neuraminidase protein HN, fusion protein F, and the products of the C, D and V open reading frames (ORFs). To the extent that the recombinant virus remains viable and infectious, each of these proteins can be selectively deleted, substituted or rearranged, in whole or in part, alone or in combination with
25 other desired modifications, to achieve novel deletion or knock out mutants. For example, one or more of the C, D, and/or V genes may be deleted in whole or in part, or its expression reduced or ablated (e.g., by introduction of a stop codon, by a mutation in an RNA editing site, by a mutation that alters the amino acid specified by an initiation codon, or by a frame shift mutation in the targeted ORF(s)). In one embodiment, a mutation can be made in the editing
30 site that prevents editing and ablates expression of proteins whose mRNA is generated by RNA editing (Kato et al., EMBO 16:578-587, 1997 and Schneider et al., Virology 227:314-322, 1997, incorporated herein by reference). Alternatively, one or more of the C, D, and/or V ORF(s) can be deleted in whole or in part to alter the phenotype of the resultant recombinant

clone to improve growth, attenuation, immunogenicity or other desired phenotypic characteristics (see, U.S. Patent Application Serial No. 09/350,821, filed by Durbin et al. on July 9, 1999, incorporated herein by reference).

Alternative nucleotide modifications in chimeric PIV of the invention include a deletion, insertion, addition or rearrangement of a cis-acting regulatory sequence for a selected gene in the recombinant genome or antigenome. In one example, a cis-acting regulatory sequence of one PIV gene is changed to correspond to a heterologous regulatory sequence, which may be a counterpart cis-acting regulatory sequence of the same gene in a different PIV, or a cis-acting regulatory sequence of a different PIV gene. For example, a gene end signal may be modified by conversion or substitution to a gene end signal of a different gene in the same PIV strain. In other embodiments, the nucleotide modification may comprise an insertion, deletion, substitution, or rearrangement of a translational start site within the recombinant genome or antigenome, e.g., to ablate an alternative translational start site for a selected form of a protein.

In addition, a variety of other genetic alterations can be produced in a chimeric PIV genome or antigenome, alone or together with one or more attenuating mutations adopted from a biologically derived mutant PIV. For example, genes or genome segments from non-PIV sources may be inserted in whole or in part. In one such aspect, the invention provides methods for attenuating chimeric PIV vaccine candidates based on host range effects due to the introduction of one or more gene(s) or genome segment(s) from, e.g., a non-human PIV into a human PIV vector-based chimeric virus. For example, host range attenuation can be conferred on a HPIV-vector based chimeric construct by introduction of nucleotide sequences from a bovine PIV (BPIV) (see, e.g., (e.g., as disclosed in United States Provisional Application Serial No. 60/143,134 filed on July 9, 1999, incorporated herein by reference). These effects are attributed to structural and functional divergence between the vector and donor viruses and provide a stable basis for attenuation. For example, between HPIV3 and BPIV3 the percent amino acid identity for each of the N proteins is 86%, for P is 65%, M 93%, F 83%, HN 77%, and L 91%. All of these proteins are therefore candidates for introduction into a HPIV vector to yield an attenuated chimeric virus which cannot readily be altered by reversion. In exemplary embodiments, the vector genome or antigenome is an HPIV3 genome or antigenome and the heterologous gene or genome segment is a N ORF derived from a selected BPIV3 strain.

In yet additional aspects of the invention, the order of genes can be changed to cause attenuation or reduce or enhance expression of a particular gene. Alternatively, a PIV genome promoter can be replaced with its antigenome counterpart to yield additional desired phenotypic changes. Different or additional modifications in the recombinant genome or antigenome can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

In yet additional aspects, polynucleotide molecules or vectors encoding the chimeric PIV genome or antigenome can be modified to encode non-PIV sequences, e.g., a cytokine, a T-helper epitope, a restriction site marker, or a protein or immunogenic epitope of a microbial pathogen (e.g., virus, bacterium or fungus) capable of eliciting a protective immune response in an intended host. In one such embodiment, chimeric PIVs are constructed that incorporate a gene encoding a cytokine to yield novel phenotypic and immunogenic effects in the resulting chimera.

In addition to providing chimeric PIV for vaccine use, the invention provides related cDNA clones and vectors which incorporate a PIV vector genome or antigenome and heterologous polynucleotide(s) encoding one or more heterologous antigenic determinants, wherein the clones and vectors optionally incorporate mutations and related modifications specifying one or more attenuating mutations or other phenotypic changes as described above. Heterologous sequences encoding antigenic determinants and/or specifying desired phenotypic changes are introduced in selected combinations, e.g., into an isolated polynucleotide which is a recombinant cDNA vector genome or antigenome, to produce a suitably attenuated, infectious virus or subviral particle in accordance with the methods described herein. These methods, coupled with routine phenotypic evaluation, provide a large assemblage of chimeric PIVs having such desired characteristics as attenuation, temperature sensitivity, altered immunogenicity, cold-adaptation, small plaque size, host range restriction, genetic stability, etc. Preferred vaccine viruses among these candidates are attenuated and yet sufficiently immunogenic to elicit a protective immune response in the vaccinated mammalian host.

In related aspects of the invention, compositions (e.g., isolated polynucleotides and vectors incorporating a chimeric PIV-encoding cDNA) and methods are provided for producing an isolated infectious chimeric PIV. Included within these aspects of the invention are novel, isolated polynucleotide molecules and vectors incorporating such molecules that

comprise a chimeric PIV genome or antigenome. Also provided is the same or different expression vector comprising one or more isolated polynucleotide molecules encoding N, P, and L proteins. These proteins can alternatively be expressed directly from the genome or antigenome cDNA. The vector(s) is/are preferably expressed or coexpressed in a cell or cell-free lysate, thereby producing an infectious chimeric parainfluenza virus particle or subviral particle.

The above methods and compositions for producing chimeric PIV yield infectious viral or subviral particles, or derivatives thereof. An infectious virus is comparable to the authentic PIV particle and is infectious as is. It can directly infect fresh cells. An infectious subviral particle typically is a subcomponent of the virus particle which can initiate an infection under appropriate conditions. For example, a nucleocapsid containing the genomic or antigenomic RNA and the N, P, and L proteins is an example of a subviral particle which can initiate an infection if introduced into the cytoplasm of cells. Subviral particles provided within the invention include viral particles which lack one or more protein(s), protein segment(s), or other viral component(s) not essential for infectivity.

In other embodiments the invention provides a cell or cell-free lysate containing an expression vector which comprises an isolated polynucleotide molecule comprising a chimeric PIV genome or antigenome as described above, and an expression vector (the same or different vector) which comprises one or more isolated polynucleotide molecules encoding the N, P, and L proteins of PIV. One or more of these proteins also can be expressed from the genome or antigenome cDNA. Upon expression the genome or antigenome and N, P and L proteins combine to produce an infectious chimeric parainfluenza virus or subviral particle.

In other embodiments of the invention a cell or cell-free expression system (e.g., a cell-free lysate) is provided which incorporates an expression vector comprising an isolated polynucleotide molecule encoding a chimeric PIV, and an expression vector comprising one or more isolated polynucleotide molecules encoding N, P, and L proteins of a PIV. Upon expression, the genome or antigenome and N, P, and L proteins combine to produce an infectious PIV particle, such as a viral or subviral particle.

The chimeric PIVs of the invention are useful in various compositions to generate a desired immune response against one or more PIVs, or against PIV and a non-PIV pathogen, in a host susceptible to infection therefrom. Chimeric PIV recombinants are capable of eliciting a mono- or poly-specific protective immune response in an infected mammalian

host, yet are sufficiently attenuated so as to not cause unacceptable symptoms of disease in the immunized host. The attenuated virus or subviral particle may be present in a cell culture supernatant, isolated from the culture, or partially or completely purified. The virus may also be lyophilized, and can be combined with a variety of other components for storage or delivery to a host, as desired.

The invention further provides novel vaccines comprising a physiologically acceptable carrier and/or adjuvant and an isolated attenuated chimeric parainfluenza virus or subviral particle as described above. In preferred embodiments, the vaccine is comprised of a chimeric PIV having at least one, and preferably two or more additional mutations or other nucleotide modifications that specify a suitable balance of attenuation and immunogenicity. The vaccine can be formulated in a dose of 10^3 to 10^7 PFU of attenuated virus. The vaccine may comprise attenuated chimeric PIV that elicits an immune response against a single PIV strain or against multiple PIV strains or groups. In this regard, chimeric PIV can be combined in vaccine formulations with other PIV vaccine strains, or with other viral vaccine viruses such as RSV.

In related aspects, the invention provides a method for stimulating the immune system of an individual to elicit an immune response against one or more PIVs, or against PIV and a non-PIV pathogen, in a mammalian subject. The method comprises administering a formulation of an immunologically sufficient amount a chimeric PIV in a physiologically acceptable carrier and/or adjuvant. In one embodiment, the immunogenic composition is a vaccine comprised of a chimeric PIV having at least one, and preferably two or more attenuating mutations or other nucleotide modifications specifying a desired phenotype and/or level of attenuation as described above. The vaccine can be formulated in a dose of 10^3 to 10^7 PFU of attenuated virus. The vaccine may comprise an attenuated chimeric PIV that elicits an immune response against a single PIV, against multiple PIVs, e.g., HPIV1 and HPIV3, or against one or more PIV(s) and a non-PIV pathogen such as measles or RSV. In this context, chimeric PIVs can elicit a monospecific immune response or a polyspecific immune response against multiple PIVs, or against one or more PIV(s) and a non-PIV pathogen. Alternatively, chimeric PIV having different immunogenic characteristics can be combined in a vaccine mixture or administered separately in a coordinated treatment protocol to elicit more effective protection against one PIV, against multiple PIVs, or against one or more PIV(s) and a non-PIV pathogen such as measles or RSV. Preferably the immunogenic compositions of the invention are administered to the upper respiratory tract, e.g., by spray, droplet or aerosol.

Preferably the immunogenic composition is administered to the upper respiratory tract, e.g., by spray, droplet or aerosol.

RSV and PIV3 cause significant amount of illness within the first four months of life, whereas most of the illness caused by PIV1 and PIV2 occurs after six months of age (Collins et al., In Fields Virology, Vol. 1, pp. 1205-1243, Lippincott-Raven Publishers, Philadelphia, 1996; Reed et al., J. Infect. Dis. 175:807-13, 1997). A preferred immunization sequence employing live attenuated RSV and PIV vaccines is to administer RSV and PIV3 as early as one month of age (e.g., at one and two months of age) followed by a bivalent PIV1 and PIV2 vaccine at four and six months of age. It is thus desirable to employ the methods of the invention to administer multiple PIV vaccines, including one or more chimeric PIV vaccines, coordinately, e.g., simultaneously in a mixture or separately in a defined temporal sequence (e.g., in a daily or weekly sequence), wherein each vaccine virus preferably expresses a different heterologous protective antigen. Such a coordinate/sequential immunization strategy, which is able to induce secondary antibody responses to multiple viral respiratory pathogens, provides a highly powerful and extremely flexible immunization regimen that is driven by the need to immunize against each of the three PIV viruses and other pathogens in early infancy.

Importantly, the presence of multiple PIV serotypes and their unique epidemiology with PIV3 disease occurring at an earlier age than that of PIV1 and PIV2 makes it desirable to sequentially immunize an infant with different PIV vectors each expressing the same heterologous antigenic determinant such as the measles virus HA. This sequential immunization permits the induction of the high titer of antibody to the heterologous protein that is characteristic of the secondary antibody response. In one embodiment, early infants (e.g. 2-4 month old infants) can be immunized with an attenuated chimeric virus of the invention, for example a chimeric HPIV3 expressing the measles virus HA protein and also adapted to elicit an immune response against HPIV3, such as *rcp45L*(HA P-M). Subsequently, e.g., at four months of age the infant is again immunized but with a different, secondary vector construct, such as the rPIV3-1 *cp45L* virus expressing the measles virus HA gene and the HPIV1 antigenic determinants as the functional, obligate glycoproteins of the vector. Following the first vaccination, the vaccinee will elicit a primary antibody response to both the PIV3 HN and F proteins and to the measles virus HA protein, but not to the PIV1 HN and F protein. Upon secondary immunization with the rPIV3-1 *cp45L* expressing the measles virus HA, the vaccinee will be readily infected with the vaccine because of the absence of antibody to the PIV1 HN and F proteins and will develop both a primary antibody response to the PIV1

HN and F protective antigens and a high titered secondary antibody response to the heterologous measles virus HA protein. A similar sequential immunization schedule can be developed where immunity is sequentially elicited against HPIV3 and then HPIV2 by one or more of the chimeric vaccine viruses disclosed herein, simultaneous with stimulation of an initial and then secondary, high titer protective response against measles or another non-PIV pathogen. This sequential immunization strategy, preferably employing different serotypes of PIV as primary and secondary vectors, effectively circumvents immunity that is induced to the primary vector, a factor ultimately limiting the usefulness of vectors with only one serotype. The success of sequential immunization with rPIV3 and rPIV3-1 virus vaccine candidates as described above has been demonstrated. (Tao et al., Vaccine 17:1100-8, 1999).

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B illustrate insertion of the HA gene of measles virus into the HPIV3 genome (Note: all of the figures presented herein and related descriptions refer to the positive-sense antigenome of HPIV3, 5' to 3').

Figure 1A provides a diagram (top; not to scale) of the 1926 nt insert containing the complete open reading frame of the hemagglutinin (HA) gene of the Edmonston wildtype strain of measles virus engineered to express the measles virus HA from an extra transcriptional unit. The insert contains, in 5' to 3' order: an *Af*/II site; nts 3699-3731 from the HPIV3 antigenome which contains the P/M gene junction, including downstream noncoding sequence for the P gene, its gene-end signal, the intergenic region, and the M gene-start signal; three additional nts (GCG); the complete measles virus HA ORF; HPIV3 nt 3594-3623 from the downstream noncoding region of the P gene; and a second *Af*/II site. Figure 1A, Panel 1 illustrates the complete antigenome of the JS wildtype strain of HPIV3 (rPIV3) with the introduced *Af*/II site in the 3'-noncoding region of the N gene before (top) and after (bottom) insertion of the measles HA ORF. Figure 1A, Panel 2 illustrates the complete antigenome of the JS wildtype strain of HPIV3 (rPIV3) with the introduced *Af*/II site in the 3'-noncoding region of the P gene before (top) and after (bottom) insertion of the measles HA ORF. SEQ ID NO: 1 and SEQ ID NO: 2 are shown in Fig. 1A.

Figure 1B provides a diagram (top; not to scale) of the 2028 nt insert containing the complete ORF of the HA gene of measles virus. The insert contains, in 5' to 3' order: a *Stu*I

site; nts 8602 to 8620 from the HPIV3 antigenome, which consist of downstream noncoding sequence from the HN gene and its gene-end signal; the conserved HPIV3 intergenic trinucleotide; nts 6733 to 6805 from the HPIV3 antigenome, which contains the HN gene-start and upstream noncoding region; the measles virus HA ORF; HPIV3 nts 8525-8597, which are downstream noncoding sequences from the HN gene; and a second *StuI* site. The construction is designed to, upon insertion, regenerate the HPIV3 HN gene containing the *StuI* site, and place the measles virus ORF directly after it flanked by the transcription signals and noncoding region of the HPIV3 HN gene. The complete antigenome of HPIV3 JS wildtype (rPIV3) with the introduced *StuI* site at nt position 8600 in the 3'-noncoding region of the HN gene is illustrated in the next (middle) diagram. Below is the antigenome of HPIV3 expressing the measles HA protein inserted into the *StuI* site. The HA cDNA used for this insertion came from an existing plasmid, rather than from the Edmonston wild type measles virus, which was used for the insertions in the N/P and P/M regions. This cDNA had two amino acid differences from the HA protein inserted in Fig 1A, and their location in the HA gene of measles virus is indicated by the asterisks in Figure 1B. SEQ ID NO: 3 and SEQ ID NO: 4 are shown in Fig. 1B.

Figure 2 illustrates expression of the HA protein of measles virus by rHPIV3-measles virus-HA chimeric viruses in LLC-MK2 cells. The figure presents a radioimmunoprecipitation assay (RIPA) demonstrating that the measles HA protein is expressed by the recombinant chimeric viruses *rcp45L*(HA P-M) and *rcp45L*(HA N-P), and by the Edmonston wild type strain of measles virus (Measles), but not by the rJS wild type HPIV3 (rJS). Lanes A--³⁵S-labeled infected cell lysates were immunoprecipitated by a mixture of three monoclonal antibodies specific to the HPIV3 HN protein). The 64kD band corresponding to the HN protein (open arrow) is present in each of the three HPIV3 infected cell lysates (lanes 3, 5, and 7), but not in the measles virus infected cell lysates (lane 9), confirming that the *rcp45L*(HA P-M) and *rcp45L*(HA N-P) chimeras are indeed HPIV3 and express similar levels of HN proteins. Lanes (b)--³⁵S-labeled infected cell lysates were immunoprecipitated by a mixture of monoclonal antibodies which recognizes the HA glycoprotein of measles virus (79-XV-V17, 80-III-B2, 81-1-366) (Hummel et al., *J. Virol.* 69:1913-6, 1995; Sheshberadaran et al., *Arch. Virol.* 83:251-68, 1985, each incorporated herein by reference). The 76kD band corresponding to the HA protein (closed arrow) is present in lysates from cells infected with the *rcp45L*(HA) chimeric viruses (lanes 6, 8) and the measles virus (lane 10), but not in the lysates from rJS infected cells (lane 4), a HPIV3 wild type virus which does not encode a measles virus HA gene.

Figure 3 illustrates insertion of the HPIV2 HN gene as an extra transcription/translation unit into the antigenomic cDNA encoding rPIV3-1 or rPIV3-1cp45 chimeric virus (Note: rPIV3-1 is a rPIV3 in which the HN and F genes were replaced by those of HPIV1, and rPIV3-1cp45 is a version which contains, in addition, 12 mutations from the cp45 attenuated virus). The HPIV2 HN gene was amplified from vRNA of HPIV2 using RT-PCR with HPIV2 HN gene specific primers (Panel A). The amplified cDNA, carrying a primer-introduced *NcoI* site at its 5'-end and a *HindIII* site at its 3'-end, was digested with *NcoI-HindIII* and ligated into pLit.PIV31HNhc, that had been digested with *NcoI-HindIII*, to generate pLit.PIV32HNhc (Panel B). The pLit.PIV32HNhc plasmid was used as a template to produce a modified PIV2 HN cassette (Panel C), which has a *PpuMI* site at its 5'-end and an introduced *PpuMI* site at its 3'-end. This cassette contained, from left to right: the *PpuMI* site at the 5'-end, a partial 5'-untranslated region (UTR) of PIV3 HN, the PIV2 HN ORF, a 3'-UTR of PIV3 HN, the gene-end, intergenic, gene-start sequence that exists at the PIV3 HN and L gene junction, a portion of the 5'-untranslated region of PIV3 L, and the introduced *PpuMI* site at the 3'-end. This cDNA cassette was digested with *PpuMI* and then ligated to p38'ΔPIV31hc, that had been digested with *PpuMI*, to generate p38'ΔPIV31hc.2HN (Panel D). The 8.5 Kb *BspEI-SphI* fragment was assembled into the *BspEI-SphI* window of pFLC.2G+.hc or pFLCcp45 to generate the final full-length antigenomic cDNA, pFLC.3-1hc.2HN (Panel E) or pFLC.3-1hc.cp45.2HN (Panel F), respectively. pFLC.2G+.hc and pFLCcp45 are full-length antigenomic clones encoding wild type rPIV3-1 and rPIV3cp45, respectively, that have been described previously (Skiadopoulos et al., *J. Virol.* 73:1374-81, 1999a; Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference).

Figure 4 details and verifies construction of the rPIV3-1.2HN chimeric virus carrying the PIV2 HN ORF insert between the PIV1 F and HN genes. Panel A depicts the differences in the structures of rPIV3-1 and rPIV3-1.2HN, which contains the PIV2 HN ORF insert between the PIV1 F and HN ORFs of rPIV3-1. The arrows indicate the approximate locations of the RT-PCR primers used to amplify fragments analyzed in Panels B-D. Panels B and C depict the expected sizes of the restriction enzyme digestion fragments generated from the RT-PCR products amplified from rPIV3-1 and rPIV3-1.2HN using either the *PpuMI* or *NcoI* restriction endonucleases, with the fragment sizes in base pairs (bp) indicated, and the results presented in panel D. vRNA extracted from virus harvested from rPIV3-1.2HN or from rPIV3-1 infected LLC-MK2 cells was used as a template in the presence and absence of reverse transcriptase (RT) to amplify cDNA fragments by PCR using primers indicated in panel A. PCR fragments were absent in RT-PCR reactions lacking RT indicating that the

template employed for amplification of the DNA fragments was RNA and not contaminating cDNA (Lanes A and C of panel D). When the RT step was included, rPIV3-1.2HN vRNA (Lane B) yielded a fragment that was approximately 2kb larger than that of its rPIV3-1 parent (Lane D) indicating the presence of an insert of 2kb. Furthermore, digestion of this 3kb

5 fragment with several different restriction endonucleases indicated that the RT-PCR fragment from rPIV3-1.2HN (odd numbered lanes) has patterns that are different from those of the rPIV3-1 parent (even numbered lanes) for each restriction endonuclease tested. For each digestion, the number of sites and the sizes of the fragments obtained were completely consistent with the predicted sequence of the RT-PCR products of rPIV3-1 and rPIV3-1.2HN. 10 Representative examples are presented. First, the *Ppu*MI digestion of the RT-PCR product from rPIV3-1.2HN (Lane 1) produced three bands of the expected sizes indicating the presence of two *Ppu*MI sites and *Ppu*MI digestion of the RT-PCR product from rPIV3-1 produced two bands of the expected sizes for rPIV3-1 (Lane 2) indicating the presence of just one *Ppu*MI site. Second, the *Nco*I digestion of the RT-PCR product from rPIV3-1.2HN (Lane 5) produced 15 4 bands including the 0.5 kb fragment indicative of the HPIV2 HN gene and the *Nco*I digestion of the RT-PCR product from rPIV3-1 (Lane 6) produced the expected two fragments. M identifies the lane containing the 1 kb DNA ladder used as nucleotide (nt) size markers (Life Technology). Similar results confirmed the presence of the HPIV2 HN insert in rPIV3-1cp45.2HN.

20 Figure 5 demonstrates that rPIV3-1.2HN expresses the HPIV2 HN protein. LLC-MK2 monolayers were infected with rPIV3-1, rPIV3-1.2HN, or the PIV2/V94 wild type virus at a MOI of 5. Infected monolayers were incubated at 32°C and labeled with ³⁵S-met and ³⁵S-cys mixture from 18-36 hours post-infection. Cells were harvested and lysed, and the proteins were immunoprecipitated with anti-HPIV2 HN mAb 150S1 (Durbin et al., Virology 25 261:319-330, 1999; Tsurudome et al., Virology 171:38-48, 1989, incorporated herein by reference) Immunoprecipitated samples were denatured, separated on a 4-12% SDS PAGE gel, and autoradiographed (Lanes: 1, rPIV3-1; 2, rPIV3-1.2HN; 3, PIV2/V9412-6). The mAb, specific to HPIV2 HN, precipitated a protein from both rPIV3-1.2HN and PIV2/V94 infected LLC-MK2 cells, but not from rPIV3-1-infected cells, with a size expected for the 86kD Kd HN 30 protein of HPIV2 (Rydbeck et al., J. Gen. Virol. 69:931-5, 1988, incorporated herein by reference).

Sub. a1
~~Figure 6 illustrates construction of the PIV3-PIV2 chimeric antigenomic cDNA pFLC.PI32he (SEQ ID NO: xxx) encoding the full-length PIV2 HN and F proteins. The~~

cDNA fragment containing the full-length PIV2 F ORF flanked by the indicated restriction sites (A1) was amplified from PIV2/V94 vRNA using RT-PCR and a PIV2 F specific primer pair (1, 2 in Table 9). This fragment was digested with NcoI plus BamHI (C1) and ligated to the NcoI-BamHI window of pLit.PIV31.fhc (B1) to generate pLit.PIV32Fhc (D1). In parallel, the cDNA fragment containing the full-length PIV2 HJN ORF flanked by the indicated restriction sites (A2) was amplified from PIV2/V94 vRNA using RT-PCR and a PIV2 HN specific primer pair (3, 4 in Table 9). This fragment was digested with NcoI plus HindIII (C2) and ligated to the NcoI-HindIII window of pLit.PIV31.HNhc (B2) to generate pLit.PIV32HNhc (D2). pLit.PIV32Fhc and pLit.PIV32HNhc were digested with PpuMI and SpeI and assembled together to generate pLit.PIV32hc (E). pLit.PIV32hc was further digested with BspEI and SpeI and introduced into the BspEI-SpeI window of p38'ΔPIV31hc (F) to generate p38'ΔPIC32hc (G). the chimeric PIV3-PIV2 construct was introduced into the BspEI-SphI window of pFLC.2G+hc to generate pFLC.PIC32hc (H).

~~Figure 7 depicts construction of full-length PIV3-PIV2 chimeric antigenomic~~

cDNA pFLC.PIV32TM (SEQ ID NO: XX) and pFLC.PIV32TMcp45, which encode F and HN proteins containing PIV2-derived ectodomains and PIV3-derived transmembrane and cytoplasmic domains. The region of the PIV3 F ORF, in pLit.PIV3.F3a (A1), encoding the ectodomain was deleted (C1) by PCR using a PIV3 F specific primer pair (9, 10 in Table 9). The region of the PIV2 F ORF encoding the ectodomain was amplified from pLit.PIV32Fhc (B1) using PCR and PIV2 F specific primer pair (5, 6 in Table 9). The two resulting fragments (C1 and D1) were ligated to generate pLit.PIV32FTM (E1). In parallel, the region of the PIV3 HN ORF, in pLit.PIV3.HN4 (A2), encoding the ectodomain was deleted (C2) by PCR using a PIV3 HN specific primer pair (11, 12 in Table 9). The region of the PIV2 HN ORF encoding the ectodomain was amplified from pLit.PIV32HNhc (B2) by PCR and a PIV2 HN specific primer pair (8, 9 in Table 9). Those two DNA fragments (C2 and D2) were ligated together to generate pLit.PIV32HNTM (E2). pLit.PIV32FTM and pLit.PIV32HNTM were digested with PpuMI and SpeI and assembled to generate pLit.PIV32TM (F). The BspEI-SpeI fragment from pLit.PIV32TM was ligated to the BspEI-SpeI window of p38' _PIV31hc (G) to generate p38' _PIV32TM (H). The insert containing chimeric PIV3-PIV2 F and HN was introduced as a 6.5 kb BspEI-SphI fragment into the BspEI-SphI window of pFLC.2G+.hc and pFLCcp45 to generate pFLC.PIV32TM and pFLC.PIV32TMcp45 (I), respectively.

~~Figure 8 shows construction of full-length PIV3-PIV2 chimeric antigenomic~~

~~cDNA pFLC.PIV32CT (SEQ ID NO: XX) and pFLC.PIV32Ctcp45 which encode F and HN~~

proteins containing a PIV2-derived ectodomain, a PIV2-derived transmembrane domain, and a PIV3-derived cytoplasmic domain. The region of the PIV3 F ORF in pLit.PIV3.F3a (A1) encoding the ectodomain and the transmembrane domain was deleted (C1) by PCR using a PIV3 F specific primer pair (17, 18 in Table 9). The region of the PIV2 F ORF encoding the ectodomain plus the transmembrane domain was amplified from pLit.PIV32Fhc (B1) using PCR and a PIV2 F specific primer pair (13, 14 in Table 9). The two resulting fragments (C1 and D1) were ligated to generate pLit.PIV32FCT (E1). In parallel, the region of the PIV3 HN ORF in pLit.PIV3.HN4 (A2), encoding the ectodomain and transmembrane domain was deleted (C2) by PCR using a PIV3 HN specific primer pair (19, 20 in Table 9). The region of the PIV2 HN ORF encoding the ectodomain plus the transmembrane domain was amplified from pLit.PIV32HNhc (B2) by PCR using a PIV2 HN specific primer pair (15, 16 in Table 9). Those two DNA fragments (C2 and D2) were ligated to generate pLit.PIV32HNCT (E2). pLit.PIV32FCT and pLit.PIV32HNCT were digested with PpuMI and SpeI and assembled to generate pLit.PIV32CT (F). The BspEI-SpeI fragment from pLit.PIV32CT was ligated to the BspEI-SpeI window of p38' _PIV31hc (G) to generate p38' _PIV32CT (H). The insert containing chimeric PIV3-PIV2 F and HN was introduced as a 6.5 kb BspEI-SphI fragment into the BspEI-SphI window of pFLC.2G+.hc and pFLC.cp45 to generate pFLC.PIV32CT and pFLC.PIV32CTcp45 (I), respectively.

Figure 9 details genetic structures of the PIV3-PIV2 chimeric viruses and the gene junction sequences for rPIV3-2CT and rPIV3-2TM. Panel A illustrates the genetic structures of rPIV3-2 chimeric viruses (middle three diagrams) are compared with that of rPIV3 (top diagram) and rPIV3-1 (bottom diagram) viruses. The cp45 derivatives are shown marked with arrows depicting the relative positions of cp45 mutations. For the cp45 derivatives, only the F and HN genes are different while the remaining genes remained identical, all from PIV3. From top to bottom, the three chimeric PIV3-PIV2 viruses carry decreasing amount of PIV3 glycoprotein genes. Note that rPIV3-2, carrying the complete PIV2 HN and F ORF, was not recoverable. Panel B provides the nucleotide sequences of the junctions of the chimeric F and HN glycoprotein genes for rPIV3-2TM are given along with the protein translation. The shaded portions represent sequences from PIV2. The amino acids are numbered with respect to their positions in the corresponding wild type glycoproteins. Three extra nucleotides were inserted in PIV3-PIV2 HN TM as indicated to make the construct conform to rule of six. Panel C shows the nucleotide sequences of the junctions of the chimeric F and HN glycoprotein genes for rPIV3-2CT, given along with the protein translation. The shaded portions represent sequences from PIV2. The amino acids are

Sub. 24
numbered with respect to their positions in the corresponding wild type glycoproteins. GE= gene end; I= intergenic; GS= gene start; ORF= open reading frame; TM= transmembrane domain; CT= cytoplasmic domain; *= stop codon. The Figure includes SEQ ID NOs: xx, xx, xx, xx.

5 Figure 10 documents multicycle replication of rPIV3-2 chimeric viruses compared with that of rPIV3/JS and PIV2/V94 wild type parent viruses. Panel A—the rPIV3-2TM and rPIV3-2TM_{cp45} viruses, along with the rPIV3/JS and PIV2/V94 wt parent viruses, were used to infect LLC-MK2 cells in 6 well plates, each in triplicate, at an MOI of 0.01. All cultures were incubated at 32°C. After a 1 hour adsorption period, the inocula were removed,
10 and the cells were washed three times with serum-free OptiMEM. The cultures were overlaid with 2 ml per well of the same medium. For rPIV3-2TM and rPIV3-2TM_{cp45} infected plates, 0.5 mg/ml of p-trypsin was added to each well. Aliquots of 0.5 ml were taken from each well at 24 hour intervals for 6 days, flash frozen on dry ice, and stored at -80°C. Each aliquot was replaced with 0.5 ml of fresh medium with or without p-trypsin as indicated
15 above. The virus present in the aliquots was titered on LLC-MK2 plates with liquid overlay at 32°C for 7 days, and the endpoints were identified with hemadsorption. Panel B—The rPIV3-2CT and rPIV3-2CT_{cp45}, along with the rPIV3/JS and PIV2/V94 wt parent viruses, were used to infect LLC-MK2 in 6 well plates, each in triplicate, as described in Panel A. Aliquots were taken and processed in the same manner as described in Panel A. Virus titers are expressed as
20 log₁₀TCID₅₀/ml ± standard errors for both experiments presented in Panel A and B.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The instant invention provides methods and compositions for the production and use of novel, chimeric parainfluenza viruses (PIVs) and associated vaccines. The chimeric
25 viruses of the invention are infectious and immunogenic in humans and other mammals and are useful for generating immune responses against one or more PIVs, for example against one or more human PIVs (HPIVs). Alternatively, chimeric PIVs are provided that elicit an immune response against a selected PIV and one or more additional pathogens, for example against both a HPIV and measles virus. The immune response elicited can involve either or both
30 humoral and/or cell mediated responses. Preferably, chimeric PIVs of the invention are attenuated to yield a desired balance of attenuation and immunogenicity for vaccine use.

The invention thus provides novel methods for designing and producing attenuated, chimeric PIVs that are useful as vaccine agents for preventing and/or treating infection and related disease symptoms attributable to PIV and other pathogens. In accordance with the methods of the invention, chimeric parainfluenza viruses or subviral particles are constructed using a PIV "vector" genome or antigenome that is recombinantly modified to incorporate one or more antigenic determinants of a heterologous pathogen. The vector genome or antigenome is comprised of a partial or complete PIV genome or antigenome, which may itself incorporate nucleotide modifications such as attenuating mutations. The vector genome or antigenome is modified to form a chimeric structure through incorporation of a heterologous gene or genome segment. More specifically, chimeric PIVs of the invention are constructed through a cDNA-based virus recovery system that yields recombinant viruses that incorporate a partial or complete vector or "background" PIV genome or antigenome combined with one or more "donor" nucleotide sequences encoding the heterologous antigenic determinant(s). Preferably the PIV vector comprises a HPIV genome or antigenome, although non-human PIVs, for example a bovine PIV (BPIV), can be employed as a vector to incorporate antigenic determinants of human PIVs and other human pathogens. In exemplary embodiments described herein, a human PIV3 (HPIV3) vector genome or antigenome is modified to incorporate one or more genes or genome segments that encode antigenic determinant(s) of one or more heterologous PIVs (e.g., HPIV1 and/or HPIV2), and/or a non-PIV pathogen (e.g., measles virus). Thus constructed, chimeric PIVs of the invention may elicit an immune response against a specific PIV, e.g., HPIV1, HPIV2, and/or HPIV3, or against a non-PIV pathogen. Alternatively, compositions and methods are provided for eliciting a polyspecific immune response against multiple PIVs, e.g., HPIV1 and HPIV3, or against one or more HPIVs and a non-PIV pathogen such as measles virus.

Exemplary chimeric PIV of the invention incorporate a chimeric PIV genome or antigenome as described above, as well as a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), and a large polymerase protein (L). Additional PIV proteins may be included in various combinations to provide a range of infectious subviral particles, up to a complete viral particle or a viral particle containing supernumerary proteins, antigenic determinants or other additional components. Additional PIV proteins may be included in various combinations to provide a range of infectious subviral particles, up to a complete viral particle or a viral particle containing supernumerary proteins, antigenic determinants or other additional components.

In preferred aspects of the invention, chimeric PIV incorporate a partial or complete human PIV vector genome or antigenome combined with one or more heterologous gene(s) or genome segment(s) from a second human PIV or a non-PIV pathogen such as measles virus. The PIV "vector" genome or antigenome typically acts as a recipient or carrier to which are added or incorporated one or more "donor" genes or genome segments of a heterologous pathogen. Typically, polynucleotides encoding one or more antigenic determinants of the heterologous pathogen are added to or substituted within the vector genome or antigenome to yield a chimeric PIV that thus acquires the ability to elicit an immune response in a selected host against the heterologous pathogen. In addition, the chimeric virus may exhibit other novel phenotypic characteristics compared to one or both of the vector PIV and heterologous pathogens.

The partial or complete vector genome or antigenome generally acts as a backbone into which heterologous genes or genome segments of a different pathogen are incorporated. Often, the heterologous pathogen is a different PIV from which one or more gene(s) or genome segment(s) is/are of are combined with, or substituted within, the vector genome or antigenome. In addition to providing novel immunogenic characteristics, the addition or substitution of heterologous genes or genome segments within the vector PIV strain may confer an increase or decrease in attenuation, growth changes, or other desired phenotypic changes as compared with the corresponding phenotype(s) of the unmodified vector and donor viruses. Heterologous genes and genome segments from other PIVs that may be selected as inserts or additions within chimeric PIV of the invention include genes or genome segments encoding the PIV N, P, C, D, V, M, F, HN and/or L protein(s) or one or more antigenic determinant(s) thereof.

Heterologous genes or genome segments of one PIV may be added as a supernumerary genomic element to a partial or complete genome or antigenome of a different PIV. Alternatively, one or more heterologous gene(s) or genome segment(s) of one PIV may be substituted at a position corresponding to a wild-type gene order position of a counterpart gene(s) or genome segment(s) that is deleted within the PIV vector genome or antigenome. In yet additional embodiments, the heterologous gene or genome segment is added or substituted at a position that is more promoter-proximal or promoter-distal compared to a wild-type gene order position of the counterpart gene or genome segment within the vector genome or antigenome to enhance or reduce, respectively, expression of the heterologous gene or genome segment.

The introduction of heterologous immunogenic proteins, protein domains and immunogenic epitopes to produce chimeric PIV is particularly useful to generate novel immune responses in an immunized host. Addition or substitution of an immunogenic gene or genome segment from one, donor pathogen within a recipient PIV vector genome or antigenome can generate an immune response directed against the donor pathogen, the PIV vector, or against both the donor pathogen and vector.

To achieve this purpose, chimeric PIV may be constructed that express a chimeric protein, for example an immunogenic glycoprotein having a cytoplasmic tail and/or transmembrane domain specific to a vector fused to a heterologous ectodomain of a different PIV or non-PIV pathogen to provide a fusion protein that elicits an immune response against the heterologous pathogen. For example, a heterologous genome segment encoding a glycoprotein ectodomain from a human PIV1 HN or F glycoprotein may be joined with a genome segment encoding the corresponding HPIV3 HN or F glycoprotein cytoplasmic and transmembrane domains to form a HPIV3-1 chimeric glycoprotein that elicits an immune response against HPIV1.

Briefly, PIV of the invention expressing a chimeric glycoprotein comprise a major nucleocapsid (N) protein, a nucleocapsid phosphoprotein (P), a large polymerase protein (L), and a HPIV vector genome or antigenome that is modified to encode a chimeric glycoprotein. The chimeric glycoprotein incorporates one or more heterologous antigenic domains, fragments, or epitopes of a second, antigenically distinct HPIV. Preferably, this is achieved by substitution within the HPIV vector genome or antigenome of one or more heterologous genome segments of the second HPIV that encode one or more antigenic domains, fragments, or epitopes, whereby the genome or antigenome encodes the chimeric glycoprotein that is antigenically distinct from the parent, vector virus.

In more detailed aspects, the heterologous genome segment or segments preferably encode a glycoprotein ectodomain or immunogenic portion or epitope thereof, and optionally include other portions of the heterologous or "donor" glycoprotein, for example both an ectodomain and transmembrane region that are substituted for counterpart glycoprotein ecto- and transmembrane domains in the vector genome or antigenome. Preferred chimeric glycoproteins in this context may be selected from HPIV HN and/or F glycoproteins, and the vector genome or antigenome may be modified to encode multiple chimeric glycoproteins. In preferred embodiments, the HPIV vector genome or antigenome is a partial HPIV3 genome or

antigenome and the second, antigenically distinct HPIV is either HPIV1 or HPIV2. In one exemplary embodiment described below, both glycoprotein ectodomain(s) of HPIV2 HN and F glycoproteins are substituted for corresponding HN and F glycoprotein ectodomains in the HPIV3 vector genome or antigenome. In another exemplary embodiment, PIV2 ectodomain and transmembrane regions of one or both HN and/or F glycoproteins are fused to one or more corresponding PIV3 cytoplasmic tail region(s) to form the chimeric glycoprotein.

To construct chimeric PIVs of the invention carrying a heterologous antigenic determinant of a non-PIV pathogen, a heterologous gene or genome segment of the donor pathogen may be added or substituted at any operable position in the vector genome or antigenome. In one embodiment, heterologous genes or genome segments from a non-PIV pathogen can be added (i.e., without substitution) within a PIV vector genome or antigenome to create novel immunogenic properties within the resultant clone. In these cases, the heterologous gene or genome segment may be added as a supernumerary gene or genome segment, optionally for the additional purpose of attenuating the resultant chimeric virus, in combination with a complete PIV vector genome or antigenome. Alternatively, the heterologous gene or genome segment may be added in conjunction with deletion of a selected gene or genome segment in the vector genome or antigenome.

In preferred embodiments of the invention, the heterologous gene or genome segment is added at an intergenic position within the partial or complete PIV vector genome or antigenome. Alternatively, the gene or genome segment can be inserted within other noncoding regions of the genome, for example, within 5' or 3' noncoding regions or in other positions where noncoding nucleotides occur within the vector genome or antigenome. In one aspect, the heterologous gene or genome segment is inserted at a non-coding site overlapping a cis-acting regulatory sequence within the vector genome or antigenome, e.g., within a sequence required for efficient replication, transcription, and/or translation. These regions of the vector genome or antigenome represent target sites for disruption or modification of regulatory functions associated with introduction of the heterologous gene or genome segment.

As used herein, the term "gene" generally refers to a portion of a subject genome, e.g., a PIV genome, encoding an mRNA and typically begins at the upstream end with a gene-start (GS) signal and ends at the downstream end with the gene-end (GE) signal. The term gene is also interchangeable with the term "translational open reading frame", or ORF, particularly in the case where a protein, such as the PIV C protein, is expressed from an

additional ORF rather than from a unique mRNA. In the exemplary case of HPIV3, the genome is a single strand of negative-sense RNA 15462 nucleotides (nt) in length (Galinski et al., Virology 165: 499-510, (1988); Stokes et al., Virus Res. 25:91-103 (1992)). At least eight proteins are encoded by the HPIV3 genome: the nucleocapsid protein N, the phosphoprotein P, the C and D proteins of unknown functions, the matrix protein M, the fusion glycoprotein F, the hemagglutinin-neuraminidase glycoprotein HN, and the large polymerase protein L (Collins et al., 3rd ed. In "Fields Virology," B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds., Vol. 1, pp. 1205-1243. Lippincott-Raven Publishers, Philadelphia, 1996). The viral genome of all PIVs also contains extragenic leader and trailer regions, possessing all or part of the promoters required for viral replication and transcription, as well as non-coding and intergenic regions. Thus, the PIV genetic map is represented as 3' leader-N-P/C/D/V-M-F-HN-L-5' trailer. Transcription initiates at the 3' end and proceeds by a sequential stop-start mechanism that is guided by short conserved motifs found at the gene boundaries. The upstream end of each gene contains a gene-start (GS) signal, which directs initiation of its respective mRNA. The downstream terminus of each gene contains a gene-end (GE) motif which directs polyadenylation and termination. Exemplary genome sequences have been described for the human PIV3 strains JS (GenBank accession number Z11575, incorporated herein by reference) and Washington (Galinski M.S. In Kingsbury, D.W. (Ed.), the Parayxoviruses, pp. 537-568, Plenum Press, New York, 1991, incorporated herein by reference), and for the bovine PIV3 strain 910N (GenBank accession number D80487, incorporated herein by reference).

To construct chimeric PIVs of the invention, one or more PIV gene(s) or genome segment(s) may be deleted, inserted or substituted in whole or in part. This means that partial or complete deletions, insertions and substitutions may include open reading frames and/or cis-acting regulatory sequences of any one or more of the PIV genes or genome segments. By "genome segment" is meant any length of continuous nucleotides from the PIV genome, which might be part of an ORF, a gene, or an extragenic region, or a combination thereof. When a subject genome segment encodes an antigenic determinant, the genome segment encodes at least one immunogenic epitope capable of eliciting a humoral or cell mediated immune response in a mammalian host. The genome segment may also encode an immunogenic fragment or protein domain. In other aspects, the donor genome segment may encode multiple immunogenic domains or epitopes, including recombinantly synthesized sequences that comprise multiple, repeating or different, immunogenic domains or epitopes.

Alternative chimeric PIV of the invention will contain protective antigenic determinants of HPIV1, HPIV2 and/or HPIV3. This is preferably achieved by expression of one or more HN and/or F genes or genome segments by the vector PIV, or as extra or substitute genes from the heterologous donor pathogen. In certain embodiments, a HPIV3-1 or HPIV3-2 chimeric virus may be constructed for use as a vaccine or vector strain, in which the HPIV1 or HPIV2 HN and/or F genes replace their PIV3 counterpart(s) (Skiadopoulos et al., Vaccine In press, 1999; Tao et al., Vaccine 17:1100-1108, 1999; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998, each incorporated herein by reference). In this context, a chimeric PIV1 vaccine candidate has been generated using the PIV3 cDNA rescue system by replacing the PIV3 HN and F open reading frames (ORFs) with those of PIV1 in a PIV3 full-length cDNA that contains the three attenuating mutations in L. The recombinant chimeric virus derived from this cDNA is designated rPIV3-1.*cp45L* (Skiadopoulos et al., J Virol 72:1762-8, 1998; Tao et al., J Virol 72:2955-2961, 1998; Tao et al., Vaccine 17:1100-1108, 1999, incorporated herein by reference). rPIV3-1.*cp45L* is attenuated in hamsters and induced a high level of resistance to challenge with PIV1. A recombinant chimeric virus, designated rPIV3-1.*cp45*, has also been produced that contains 12 of the 15 *cp45* mutations, i.e., excluding the mutations in HN and F, and is highly attenuated in the upper and lower respiratory tract of hamsters (Skiadopoulos et al., Vaccine 18:503-510, 1999, incorporated herein by reference).

In preferred embodiments of the invention, the chimeric PIV bear one or more major antigenic determinants of a human PIV, or against multiple human PIVs, including HPIV1, HPIV2 or HPIV3. These preferred vaccine candidates elicit an effective immune response in humans against one or more selected HPIVs. As noted above, the antigenic determinant(s) that elicit(s) an immune response against HPIV may be encoded by the vector genome or antigenome, or may be inserted within or joined to the PIV vector genome or antigenome as a heterologous gene or gene segment. The major protective antigens of human PIVs are their HN and F glycoproteins. However, all PIV genes are candidates for encoding antigenic determinants of interest, including internal protein genes which may encode such determinants as, for example, CTL epitopes.

Preferred chimeric PIV vaccine viruses of the invention bear one or more major antigenic determinants from each of a plurality of HPIVs or from a HPIV and a non-PIV pathogen. Chimeric PIV thus constructed include a partial or complete HPIV genome or antigenome, for example of HPIV3, and one or more heterologous gene(s) or genome segment(s) encoding antigenic determinant(s) of a heterologous PIV, for example HPIV1 or

HPIV2. In alternative embodiments, one or more genes or genome segments encoding one or more antigenic determinants of HPIV1 or HPIV2 may be added to or substituted within a partial or complete HPIV3 genome or antigenome. In various exemplary embodiments described below, both HPIV1 genes encoding the HN and F glycoproteins are substituted for counterpart HPIV3 HN and F genes in a chimeric PIV vaccine candidate. These and other constructs yield chimeric PIVs that elicit either a mono- or poly-specific immune response in humans to one or more HPIVs. Further detailed aspects of the invention are provided in United States Patent Application entitled USE OF RECOMBINANT PARAINFLUENZA VIRUS (PIV) AS A VECTOR TO PROTECT AGAINST DISEASE CAUSED BY PIV AND RESPIRATORY SYNCYTIAL VIRUS (RSV), filed on December 10, 1999 by Murphy et al. and identified by Attorney Docket No. 17634-000330, and U.S. Provisional Patent Application entitled USE OF RECOMBINANT PARAINFLUENZA VIRUSES (PIVs) AS VECTORS TO PROTECT AGAINST INFECTION AND DISEASE CAUSED BY PIV AND OTHER HUMAN PATHOGENS, filed on December 10, 1999 by Murphy et al. and identified by Attorney Docket No. 152800-404000, each incorporated herein by reference.

In exemplary aspects of the invention, heterologous genes or genome segments encoding antigenic determinants from both HPIV1 and HPIV2 are added to or incorporated within a partial or complete HPIV3 vector genome or antigenome. For instance, one or more HPIV1 genes or genome segments encoding HN and/or F glycoproteins, or antigenic determinant(s) thereof, and one or more HPIV2 genes or genome segments encoding HN and/or F glycoproteins or antigenic determinants can be added to or incorporated within a partial or complete HPIV3 vector genome or antigenome. In one example described below, both HPIV1 genes encoding HN and F glycoproteins are substituted for counterpart HPIV3 HN and F genes to form a chimeric HPIV3-1 vector genome or antigenome. This vector construct can be further modified by addition or incorporation of one or more genes or gene segments encoding antigenic determinant(s) of HPIV2. Thus, specific constructs exemplifying the invention are provided which yield chimeric PIVs having antigenic determinants of both HPIV1 and HPIV2, as exemplified by the vaccine candidates rPIV3-1.2HN and rPIV3-1cp45.2HN described herein below.

In other preferred aspects of the invention, chimeric PIV incorporate a HPIV vector genome or antigenome modified to express one or more major antigenic determinants of non-PIV pathogen, for example measles virus. The methods of the invention are generally adaptable for incorporation of antigenic determinants from a wide range of additional

pathogens within chimeric PIV vaccine candidates. In this regard the invention also provides for development of vaccine candidates against subgroup A and subgroup B respiratory syncytial viruses (RSV), mumps virus, human papilloma viruses, type 1 and type 2 human immunodeficiency viruses, herpes simplex viruses, cytomegalovirus, rabies virus, Epstein Barr virus, filoviruses, bunyaviruses, flaviviruses, alphaviruses and influenza viruses, among other pathogens. In this regard, pathogens that may be targeted for vaccine development according to the methods of the invention include viral and bacterial pathogens, as well as protozoans and multicellular pathogens. Useful antigenic determinants from many important human pathogens in this context are known or readily identified for incorporation within chimeric PIV of the invention. Thus, major antigens have been identified for the foregoing exemplary pathogens, including the measles virus HA and F proteins; the F, G, SH and M2 proteins of RSV, mumps virus HN and F proteins, human papilloma virus L1 protein, type 1 or type 2 human immunodeficiency virus gp160 protein, herpes simplex virus and cytomegalovirus gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM proteins, rabies virus G protein, Epstein Barr Virus gp350 protein; filovirus G protein, bunyavirus G protein, flavivirus E and NS1 proteins, and alphavirus E. These major antigens, as well as other antigens known in the art for the enumerated pathogens and others, are well characterized to the extent that many of their antigenic determinants, including the full length proteins and their constituent antigenic domains, fragments and epitopes, are identified, mapped and characterized for their respective immunogenic activities.

Among the numerous, exemplary mapping studies that identify and characterize major antigens of diverse pathogens for use within the invention are epitope mapping studies directed to the hemagglutinin-neuraminidase (HN) gene of HPIV3. van Wyke Coelingh et al., J. Virol. 61(5):1473-1477, 1987, incorporated herein by reference. This report provides detailed antigenic structural analyses for 16 antigenic variants of HPIV3 variants selected by using monoclonal antibodies (MAbs) to the HN protein which inhibit neuraminidase, hemagglutination, or both activities. Each variant possessed a single-point mutation in the HN gene, coding for a single amino acid substitution in the HN protein. Operational and topographic maps of the HN protein correlated well with the relative positions of the substitutions. Computer-assisted analysis of the HN protein predicted a secondary structure composed primarily of hydrophobic β sheets interconnected by random hydrophilic coil structures. The HN epitopes were located in predicted coil regions. Epitopes recognized by MAbs which inhibit neuraminidase activity of the virus were located in a region which appears

to be structurally conserved among several paramyxovirus HN proteins and which may represent the sialic acid-binding site of the HN molecule.

This exemplary work, employing conventional antigenic mapping methods, identified single amino acids which are important for the integrity of HN epitopes. Most of these epitopes are located in the C-terminal half of the molecule, as expected for a protein anchored at its N terminus (Elango et al., J. Virol. 57:481-489, 1986). Previously published operational and topographic maps of the PIV3 HN indicated that the MABs employed recognized six distinct groups of epitopes (I to VI) organized into two topographically separate sites (A and B), which are partially bridged by a third site (C). These groups of epitopes represent useful candidates for antigenic determinants that may be incorporated, alone or in various combinations, within chimeric PIVs of the invention. (See, also, Coelingh et al., Virology 143:569-582, 1985; Coelingh et al., Virology 162:137-143, 1988; Ray et al., Virology 148:232-236, 1986; Rydbeck et al., J. Gen. Virol. 67:1531-1542, 1986, each incorporated herein by reference),

Additional studies by van Wyke Coelingh et al., J. Virol. 63(1):375-382, 1989, provide further information relating to selection of PIV antigenic determinants for use within the invention. In this study, twenty-six monoclonal antibodies (MABs) (14 neutralizing and 12 nonneutralizing) were used to examine the antigenic structure, biological properties, and natural variation of the fusion (F) glycoprotein of HPIV3. Analysis of laboratory-selected antigenic variants and of PIV3 clinical isolates indicated that the panel of MABs recognizes at least 20 epitopes, 14 of which participate in neutralization. Competitive binding assays confirmed that the 14 neutralization epitopes are organized into three nonoverlapping principal antigenic regions (A, B, and C) and one bridge site (AB), and that the 6 nonneutralization epitopes form four sites (D, E, F, and G). Most of the neutralizing MABs were involved in nonreciprocal competitive binding reactions, suggesting that they induce conformational changes in other neutralization epitopes.

Other antigenic determinants for use within the invention have been identified and characterized for respiratory syncytial virus (RSV). For example, Beeler et al., J. Virol. 63(7):2941-2950, 1989, incorporated herein by reference, employed eighteen neutralizing monoclonal antibodies (MABs) specific for the fusion glycoprotein of the A2 strain of RSV to construct a detailed topological and operational map of epitopes involved in RSV neutralization and fusion. Competitive binding assays identified three nonoverlapping

antigenic regions (A, B, and C) and one bridge site (AB). Thirteen MAb-resistant mutants (MARMs) were selected, and the neutralization patterns of the MAbs with either MARMs or RSV clinical strains identified a minimum of 16 epitopes. MARMs selected with antibodies to six of the site A and AB epitopes displayed a small-plaque phenotype, which is consistent with an alteration in a biologically active region of the F molecule. Analysis of MARMs also indicated that these neutralization epitopes occupy topographically distinct but conformationally interdependent regions with unique biological and immunological properties. Antigenic variation in F epitopes was then examined by using 23 clinical isolates (18 subgroup A and 5 subgroup B) in cross-neutralization assays with the 18 anti-F MAbs. This analysis identified constant, variable, and hypervariable regions on the molecule and indicated that antigenic variation in the neutralization epitopes of the RSV F glycoprotein is the result of a noncumulative genetic heterogeneity. Of the 16 epitopes, 8 were conserved on all or all but 1 of 23 subgroup A or subgroup B clinical isolates. These antigenic determinants, including the full length proteins and their constituent antigenic domains, fragments and epitopes, all represent useful candidates for integration within chimeric PIV of the invention to elicit novel immune responses as described above. (See also, Anderson et al., J. Infect. Dis. 151:626-633, 1985; Coelingh et al., J. Virol. 63:375-382, 1989; Fenner et al., Scand. J. Immunol. 24:335-340, 1986; Fernie et al., Proc. Soc. Exp. Biol. Med. 171:266-271, 1982; Sato et al., J. Gen. Virol. 66:1397-1409, 1985; Walsh et al., J. Gen. Virol. 67:505-513, 1986, and Olmsted et al., J. Virol. 63(1):411-420, 1989, each incorporated herein by reference).

To express antigenic determinants of heterologous PIVs and non-PIV pathogens, the invention provides numerous human and non-human PIV vectors, including bovine PIV (BPIV) vectors. These vectors are readily modified according the recombinant methods described herein to carry heterologous antigenic determinants and elicit one or more specific humoral or cell mediated immune responses against the heterologous pathogen and vector PIV. In exemplary embodiments, one or more heterologous genes or genome segments from a donor pathogen is combined with a HPIV3 vector genome or antigenome. In other exemplary embodiments, the heterologous gene or genome segment is incorporated within a chimeric HPIV vector genome or antigenome, for example a chimeric HPIV3-1 vector genome or antigenome having one or both HPIV1 genes encoding the HN and F glycoproteins substituted for their counterpart HPIV3 HN and/or F gene(s). In more detailed embodiments, a transcription unit comprising an open reading frame (ORF) of the measles virus HA gene is added to a HPIV3 vector genome or antigenome at various positions, yielding exemplary chimeric PIV/measles vaccine candidates rPIV3(HA HN-L), rPIV3(HA N-P), rcp45L(HA N-

P), rPIV3(HA P-M), or rcp45L(HA P-M). Alternatively, chimeric PIV for vaccine use may incorporate one or more antigenic determinants of HPIV2, for example an HPIV2 HN gene, within a chimeric HPIV3-1 vector genome or antigenome.

In other detailed embodiments of the invention, chimeric PIVs are engineered that incorporate heterologous nucleotide sequences encoding protective antigens from respiratory syncytial virus (RSV) to produce infectious, attenuated vaccine candidates. The cloning of RSV cDNA and other disclosure is provided in U.S. Provisional Patent Application No. 60/007,083, filed September 27, 1995; U.S. Patent Application No. 08/720,132, filed September 27, 1996; U.S. Provisional Patent Application No. 60/021,773, filed July 15, 1996; U.S. Provisional Patent Application No. 60/046,141, filed May 9, 1997; U.S. Provisional Patent Application No. 60/047,634, filed May 23, 1997; U.S. Patent Application No. 08/892,403, filed July 15, 1997 (corresponding to International Publication No. WO 98/02530); U.S. Patent Application No. 09/291,894, filed on April 13, 1999; U.S. Provisional Patent Application Serial No. 60/129,006, filed on April 13, 1999; Collins, et al., Proc Nat. Acad. Sci. USA 92:11563-11567, 1995; Bukreyev, et al., J Virol 70:6634-41, 1996, Juhasz et al., J. Virol. 71(8):5814-5819, 1997; Durbin et al., Virology 235:323-332, 1997; He et al. Virology 237:249-260, 1997; Baron et al. J. Virol. 71:1265-1271, 1997; Whitehead et al., Virology 247(2):232-9, 1998a; Whitehead et al., J. Virol. 72(5):4467-4471, 1998b; Jin et al. Virology 251:206-214, 1998; and Whitehead et al., J. Virol. 73:(4)3438-3442, 1999, and Bukreyev, et al., Proc Nat Acad Sci USA 96:2367-72, 1999, each incorporated herein by reference in its entirety for all purposes). Other reports and discussion incorporated or set forth herein identify and characterize RSV antigenic determinants that are useful within the invention.

PIV chimeras incorporating one or more RSV antigenic determinants, preferably comprise a human PIV (e.g., HPIV1, HPIV2, HPIV3) vector genome or antigenome with a heterologous gene or genome segment encoding an antigenic RSV glycoprotein, protein domain (e.g., a glycoprotein ectodomain) or one or more immunogenic epitopes. In one embodiment, one or more genes or genome segments from RSV F and/or G genes is/are combined with the vector genome or antigenome to form the chimeric PIV vaccine candidate. Certain of these constructs will express chimeric proteins, for example fusion proteins having a cytoplasmic tail and/or transmembrane domain of PIV fused to an ectodomain of RSV to yield a novel attenuated virus that elicits a multivalent immune response against both PIV and RSV

As noted above, it is often desirable to adjust the phenotype of chimeric PIV for vaccine use by introducing additional mutations that increase or decrease attenuation or otherwise alter the phenotype of the chimeric virus. Detailed descriptions of the materials and methods for producing recombinant PIV from cDNA, and for making and testing various mutations and nucleotide modifications set forth herein as supplemental aspects of the present invention are provided in, e.g., Durbin et al., Virology 235:323-332, 1997; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997, each incorporated herein by reference. In particular, these documents describe methods and procedures for mutagenizing, isolating and characterizing PIV to obtain attenuated mutant strains (e.g., temperature sensitive (*ts*), cold passaged (*cp*) cold-adapted (*ca*), small plaque (*sp*) and host-range restricted (*hr*) mutant strains) and for identifying the genetic changes that specify the attenuated phenotype. In conjunction with these methods, the foregoing documents detail procedures for determining replication, immunogenicity, genetic stability and protective efficacy of biologically derived and recombinantly produced attenuated human PIV in accepted model systems, including murine and non-human primate model systems. In addition, these documents describe general methods for developing and testing immunogenic compositions, including monovalent and bivalent vaccines, for prophylaxis and treatment of PIV infection. Methods for producing infectious recombinant PIV by construction and expression of cDNA encoding a PIV genome or antigenome coexpressed with essential PIV proteins are also described in the above-incorporated documents, which include description of the following exemplary plasmids that may be employed to produce infectious PIV clones: p3/7(131) (ATCC 97990); p3/7(131)2G (ATCC 97889); and p218(131) (ATCC 97991); each deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) of 10801 University Boulevard, Manassas, Virginia 20110-2209, U.S.A., and granted the above identified accession numbers.

Also disclosed in the above-incorporated references are methods for constructing and evaluating infectious recombinant PIV that are modified to incorporate phenotype-specific mutations identified in biologically-derived PIV mutants, e.g., cold passaged (*cp*), cold adapted (*ca*), host range restricted (*hr*), small plaque (*sp*), and/or temperature sensitive (*ts*) mutants, for example the JS HPIV3 *cp* 45 mutant strain. Mutations identified in these mutants can be readily incorporated into chimeric PIV of the instant invention. In exemplary embodiments, one or more attenuating mutations occur in the

polymerase L protein, e.g., at a position corresponding to Tyr₉₄₂, Leu₉₉₂, or Thr₁₅₅₈ of JS *cp45*. Preferably, these mutations are incorporated in chimeric PIV of the invention by an identical, or conservative, amino acid substitution as identified in the biological mutant. In more detailed aspects, chimeric PIV for vaccine use incorporate one or more mutation wherein Tyr₉₄₂ is replaced by His, Leu₉₉₂ is replaced by Phe, and/or Thr₁₅₅₈ is replaced by Ile. Substitutions that are conservative to these replacement amino acids are also useful to achieve desired attenuation in chimeric vaccine candidates.

Other exemplary mutations that can be adopted in chimeric PIVs from biologically derived PIV mutants include one or more mutations in the N protein, including specific mutations at a position corresponding to residues Val₉₆ or Ser₃₈₉ of JS *cp45*. In more detailed aspects, these mutations are represented as Val₉₆ to Ala or Ser₃₈₉ to Ala or substitutions that are conservative thereto. Also useful within chimeric PIV of the invention are amino acid substitution in the C protein, e.g., a mutation at a position corresponding to Ile₉₆ of JS *cp45*, preferably represented by an identical or conservative substitution of Ile₉₆ to Thr. Further exemplary mutations that can be adopted from biologically derived PIV mutants include one or more mutations in the F protein, including mutations adopted from JS *cp45* at a position corresponding to residues Ile₄₂₀ or Ala₄₅₀ of JS *cp45*, preferably represented by acid substitutions Ile₄₂₀ to Val or Ala₄₅₀ to Thr or substitutions conservative thereto. Alternatively or in addition, chimeric PIV of the invention can adopt one or more amino acid substitutions in the HN protein, as exemplified by a mutation at a position corresponding to residue Val₃₈₄ of JS *cp45*, preferably represented by the substitution Val₃₈₄ to Ala.

Yet additional embodiments of the invention include chimeric PIV which incorporate one or more mutations in noncoding portions of the PIV genome or antigenome, for example in a 3' leader sequence, that specify desired phenotypic changes such as attenuation. Exemplary mutations in this context may be engineered at a position in the 3' leader of the chimeric virus at a position corresponding to nucleotide 23, 24, 28, or 45 of JS *cp45*. Yet additional exemplary mutations may be engineered in the N gene start sequence, for example by changing one or more nucleotides in the N gene start sequence, e.g., at a position corresponding to nucleotide 62 of JS *cp45*. In more detailed aspects, chimeric PIV incorporate a T to C change at nucleotide 23, a C to T change at nucleotide 24, a G to T change at nucleotide 28, and/or a T to A change at nucleotide 45. Additional mutations in extragenic sequences are exemplified by a A to T change in the N gene start sequence at a position corresponding to nucleotide 62 of JS.

These foregoing exemplary mutations which can be engineered in a chimeric PIV of the invention have been successfully engineered and recovered in recombinant PIV—as represented by the recombinant PIV clones designated *rcp45*, *rcp45 L*, *rcp45 F*, *rcp45 M*, *rcp45 HN*, *rcp45 C*, *rcp45 F*, *rcp45 3'N*, *rcp3'NL*, and *rcp45 3'NCMFHN* (Durbin et al., Virology 235:323-332, 1997; Skiadopolos et al., J. Virol. 72:1762-1768 (1998); Skiadopolos et al., J. Virol. 73:1374-1381, 1999; U.S. Patent Application Serial No. 09/083,793, filed May 22, 1998; U.S. Provisional Application No. 60/047,575, filed May 23, 1997 (corresponding to International Publication No. WO 98/53078), and U.S. Provisional Application No. 60/059,385, filed September 19, 1997, each incorporated herein by reference). In addition, the above-incorporated references describe construction of chimeric PIV recombinants, e.g., having the HN and F genes of HPIV1 substituted into a partial HPIV3 background genome or antigenome, which is further modified to bear one or more of the attenuating mutations identified in HPIV3 JS *cp45*. One such chimeric recombinant incorporates all of the attenuating mutations identified in the L gene of *cp45*. It has since been shown that all of the *cp45* mutations outside of the heterologous (HPIV1) HN and F genes can be incorporated in a HPIV3-1 recombinant to yield an attenuated, chimeric vaccine candidate.

From JS *cp45* and other biologically derived PIV mutants, a large "menu" of attenuating mutations is provided, each of which can be combined with any other mutation(s) for adjusting the level of attenuation, immunogenicity and genetic stability in chimeric PIV of the invention. In this context, many chimeric PIVs will include one or more, and preferably two or more, mutations from biologically derived PIV mutants, e.g., any one or combination of mutations identified in JS *cp45*. Preferred chimeric PIVs within the invention will incorporate a plurality and up to a full complement of the mutations present in JS *cp45* or other biologically derived mutant PIV strains. Preferably, these mutations are stabilized against reversion in chimeric PIV by multiple nucleotide substitutions in a codon specifying each mutation.

Yet additional mutations that may be incorporated in chimeric PIV of the invention are mutations, e.g., attenuating mutations, identified in heterologous PIV or other nonsegmented negative stranded RNA viruses. In particular, attenuating and other desired mutations identified in one negative stranded RNA virus may be "transferred", e.g., copied, to a corresponding position within the genome or antigenome of a chimeric PIV. Briefly, desired mutations in one heterologous negative stranded RNA virus are transferred to the chimeric PIV recipient (either in the vector genome or antigenome or in the heterologous donor gene or

genome segment). This involves mapping the mutation in the heterologous mutant virus, identifying by routine sequence alignment the corresponding site in the recipient PIV, and mutating the native sequence in the PIV recipient to the mutant genotype (either by an identical or conservative mutation), as described in U.S. Provisional Patent Application Serial No.

5 60/129,006, filed on April 13, 1999, incorporated herein by reference. As this disclosure teaches, it is preferable to modify the recipient chimeric PIV genome or antigenome to encode an alteration at the subject site of mutation that corresponds conservatively to the alteration identified in the heterologous mutant virus. For example, if an amino acid substitution marks a site of mutation in the mutant virus compared to the corresponding wild-type sequence, then a
10 similar substitution can be engineered at the corresponding residue(s) in the recombinant virus. Preferably the substitution will specify an identical or conservative amino acid to the substitute residue present in the mutant viral protein. However, it is also possible to alter the native amino acid residue at the site of mutation non-conservatively with respect to the substitute residue in the mutant protein (e.g., by using any other amino acid to disrupt or impair the
15 function of the wild-type residue). Negative stranded RNA viruses from which exemplary mutations are identified and transferred into a recombinant PIV of the invention include other PIVs (e.g., HPIV1, HPIV2, HPIV3, BPIV and MPIV), RSV, Sendai virus (SeV), Newcastle disease virus (NDV), simian virus 5 (SV5), measles virus (MeV), rinderpest virus, canine distemper virus (CDV), rabies virus (RaV) and vesicular stomatitis virus (VSV), among others.
20 A variety of exemplary mutations are disclosed, including but not limited to an amino acid substitution of phenylalanine at position 521 of the RSV L protein corresponding to and therefore transferable to a substitution of phenylalanine (or a conservatively related amino acid) at position 456 of the HPIV3 L protein. In the case of mutations marked by deletions or insertions, these can be introduced as corresponding deletions or insertions into the
25 recombinant virus, however the particular size and amino acid sequence of the deleted or inserted protein fragment can vary.

Attenuating mutations in biologically derived PIV and other nonsegmented negative stranded RNA viruses for incorporation within chimeric PIV of the invention may occur naturally or may be introduced into wild-type PIV strains by well known mutagenesis
30 procedures. For example, incompletely attenuated parental PIV strains can be produced by chemical mutagenesis during virus growth in cell cultures to which a chemical mutagen has been added, by selection of virus that has been subjected to passage at suboptimal temperatures in order to introduce growth restriction mutations, or by selection of a mutagenized virus that produces small plaques (*sp*) in cell culture, as described in the above incorporated references.

By "biologically derived PIV" is meant any PIV not produced by recombinant means. Thus, biologically derived PIV include all naturally occurring PIV, including, e.g., naturally occurring PIV having a wild-type genomic sequence and PIV having allelic or mutant genomic variations from a reference wild-type PIV sequence, e.g., PIV having a mutation specifying an attenuated phenotype. Likewise, biologically derived PIV include PIV mutants derived from a parental PIV by, *inter alia*, artificial mutagenesis and selection procedures.

As noted above, production of a sufficiently attenuated biologically derived PIV mutant can be accomplished by several known methods. One such procedure involves subjecting a partially attenuated virus to passage in cell culture at progressively lower, attenuating temperatures. For example, partially attenuated mutants are produced by passage in cell cultures at suboptimal temperatures. Thus, a *cp* mutant or other partially attenuated PIV strain is adapted to efficient growth at a lower temperature by passage in culture. This selection of mutant PIV during cold-passage substantially reduces any residual virulence in the derivative strains as compared to the partially attenuated parent. Alternatively, specific mutations can be introduced into biologically derived PIV by subjecting a partially attenuated parent virus to chemical mutagenesis, e.g., to introduce *ts* mutations or, in the case of viruses which are already *ts*, additional *ts* mutations sufficient to confer increased attenuation and/or stability of the *ts* phenotype of the attenuated derivative. Means for the introduction of *ts* mutations into PIV include replication of the virus in the presence of a mutagen such as 5-fluorouridine according to generally known procedures. Other chemical mutagens can also be used. Attenuation can result from a *ts* mutation in almost any PIV gene, although a particularly amenable target for this purpose has been found to be the polymerase (L) gene. The level of temperature sensitivity of replication in exemplary attenuated PIV for use within the invention is determined by comparing its replication at a permissive temperature with that at several restrictive temperatures. The lowest temperature at which the replication of the virus is reduced 100-fold or more in comparison with its replication at the permissive temperature is termed the shutoff temperature. In experimental animals and humans, both the replication and virulence of PIV correlate with the mutant's shutoff temperature.

The JS *cp45* HPIV3 mutant has been found to be relatively stable genetically, highly immunogenic, and satisfactorily attenuated. Nucleotide sequence analysis of this biologically derived virus, and of recombinant viruses incorporating various individual and combined mutations found therein, indicates that each level of increased attenuation is associated with specific nucleotide and amino acid substitutions. The above-incorporated

references also disclose how to routinely distinguish between silent incidental mutations and those responsible for phenotype differences by introducing the mutations, separately and in various combinations, into the genome or antigenome of infectious PIV clones. This process coupled with evaluation of phenotype characteristics of parental and derivative viruses identifies mutations responsible for such desired characteristics as attenuation, temperature sensitivity, cold-adaptation, small plaque size, host range restriction, etc.

Mutations thus identified are compiled into a "menu" and are then introduced as desired, singly or in combination, to adjust chimeric PIV of the invention to an appropriate level of attenuation, immunogenicity, genetic resistance to reversion from an attenuated phenotype, etc., as desired. In accordance with the foregoing description, the ability to produce infectious PIV from cDNA permits introduction of specific engineered changes within chimeric PIV. In particular, infectious, recombinant PIVs are employed for identification of specific mutation(s) in biologically derived, attenuated PIV strains, for example mutations which specify *ts*, *ca*, *att* and other phenotypes. Desired mutations are thus identified and introduced into chimeric PIV vaccine strains. The capability of producing virus from cDNA allows for routine incorporation of these mutations, individually or in various selected combinations, into a full-length cDNA clone, whereafter the phenotypes of rescued recombinant viruses containing the introduced mutations to be readily determined.

By identifying and incorporating specific mutations associated with desired phenotypes, e.g., a *cp* or *ts* phenotype, into infectious chimeric PIV clones, the invention provides for other, site-specific modifications at, or within close proximity to, the identified mutation. Whereas most attenuating mutations produced in biologically derived PIVs are single nucleotide changes, other "site specific" mutations can also be incorporated by recombinant techniques into a chimeric PIV. As used herein, site-specific mutations include insertions, substitutions, deletions or rearrangements of from 1 to 3, up to about 5-15 or more altered nucleotides (e.g., altered from a wild-type PIV sequence, from a sequence of a selected mutant PIV strain, or from a parent recombinant PIV clone subjected to mutagenesis). Such site-specific mutations may be incorporated at, or within the region of, a selected, biologically derived point mutation. Alternatively, the mutations can be introduced in various other contexts within a PIV clone, for example at or near a cis-acting regulatory sequence or nucleotide sequence encoding a protein active site, binding site, immunogenic epitope, etc. Site-specific PIV mutants typically retain a desired attenuating phenotype, but may additionally exhibit altered phenotypic characteristics unrelated to attenuation, e.g., enhanced

or broadened immunogenicity, and/or improved growth. Further examples of desired, site-specific mutants include recombinant PIV designed to incorporate additional, stabilizing nucleotide mutations in a codon specifying an attenuating point mutation. Where possible, two or more nucleotide substitutions are introduced at codons that specify attenuating amino acid changes in a parent mutant or recombinant PIV clone, yielding a PIV with greater genetic resistance to reversion from an attenuated phenotype. In other embodiments, site-specific nucleotide substitutions, additions, deletions or rearrangements are introduced upstream (N-terminal direction) or downstream (C-terminal direction), e. g., from 1 to 3, 5-10 and up to 15 nucleotides or more 5' or 3', relative to a targeted nucleotide position, e.g., to construct or ablate an existing cis-acting regulatory element.

In addition to single and multiple point mutations and site-specific mutations, changes to the chimeric PIV disclosed herein include deletions, insertions, substitutions or rearrangements of one or more gene(s) or genome segment(s). Particularly useful are deletions involving one or more gene(s) or genome segment(s), which deletions have been shown to yield additional desired phenotypic effects. Thus, U.S. Patent Application Serial No. 09/350,821, filed by Durbin et al. on July 9, 1999, incorporated herein by reference, describes methods and compositions whereby expression of one or more HPIV genes, for example one or more of the C, D, and/or V ORFs, is reduced or ablated by modifying the PIV genome or antigenome to incorporate a mutation that alters the coding assignment of an initiation codon or mutation(s) that introduce one or one or more stop codon(s). Alternatively, one or more of the C, D, and/or V ORFs can be deleted in whole or in part to render the corresponding protein(s) partially or entirely non-functional or to disrupt protein expression altogether. Chimeric PIV having such mutations in C, D, and/or V, or other non-essential gene(s), possess highly desirable phenotypic characteristics for vaccine development. For example, these modifications may specify one or more desired phenotypic changes including (i) altered growth properties in cell culture, (ii) attenuation in the upper and/or lower respiratory tract of mammals, (iii) a change in viral plaque size, (iv) a change in cytopathic effect, and (v) a change in immunogenicity. One exemplary "knock out" mutant PIV lacking C ORF expression, designated rC-KO, was able to induce a protective immune response against wild type HPIV3 challenge in a non-human primate model despite its beneficial attenuation phenotype.

Thus, in more detailed aspects of the instant invention, chimeric PIV incorporate deletion or knock out mutations in a C, D, and/or V ORF(s) or other non-essential

gene which alters or ablates expression of the selected gene(s) or genome segment(s). This can be achieved, e.g., by introducing a frame shift mutation or termination codon within a selected coding sequence, altering translational start sites, changing the position of a gene or introducing an upstream start codon to alter its rate of expression, changing GS and/or GE transcription signals to alter phenotype, or modifying an RNA editing site (e.g., growth, temperature restrictions on transcription, etc.). In more detailed aspects of the invention, chimeric PIVs are provided in which expression of one or more gene(s), e.g., a C, D, and/or V ORF(s), is ablated at the translational or transcriptional level without deletion of the gene or of a segment thereof, by, e.g., introducing multiple translational termination codons into a translational open reading frame (ORF), altering an initiation codon, or modifying an editing site. These forms of knock-out virus will often exhibit reduced growth rates and small plaque sizes in tissue culture. Thus, these methods provide yet additional, novel types of attenuating mutations which ablate expression of a viral gene that is not one of the major viral protective antigens. In this context, knock-out virus phenotypes produced without deletion of a gene or genome segment can be alternatively produced by deletion mutagenesis, as described, to effectively preclude correcting mutations that may restore synthesis of a target protein. Several other gene knock-outs for the C, D, and/or V ORF(s) deletion and knock out mutants can be made using alternate designs and methods that are well known in the art (as described, for example, in (Kretschmer et al., Virology 216:309-316, 1996; Radecke et al., Virology 217:418-421, 1996; and Kato et al., EMBO J. 16:578-587, 1987; and Schneider et al., Virology 277:314-322, 1996, each incorporated herein by reference).

Nucleotide modifications that may be introduced into chimeric PIV constructs of the invention may alter small numbers of bases (e.g., from 15-30 bases, up to 35-50 bases or more), large blocks of nucleotides (e.g., 50-100, 100-300, 300-500, 500-1,000 bases), or nearly complete or complete genes (e.g., 1,000-1,500 nucleotides, 1,500-2,500 nucleotides, 2,500-5,000, nucleotides, 5,000-6,5000 nucleotides or more) in the vector genome or antigenome or heterologous, donor gene or genome segment, depending upon the nature of the change (i.e., a small number of bases may be changed to insert or ablate an immunogenic epitope or change a small genome segment, whereas large block(s) of bases are involved when genes or large genome segments are added, substituted, deleted or rearranged).

In related aspects, the invention provides for supplementation of mutations adopted into a chimeric PIV clone from biologically derived PIV, e.g., *cp* and *ts* mutations, with additional types of mutations involving the same or different genes in a further modified

PIV clone. Each of the PIV genes can be selectively altered in terms of expression levels, or can be added, deleted, substituted or rearranged, in whole or in part, alone or in combination with other desired modifications, to yield a chimeric PIV exhibiting novel vaccine characteristics. Thus, in addition to or in combination with attenuating mutations adopted from biologically derived PIV mutants, the present invention also provides a range of additional methods for attenuating or otherwise modifying the phenotype of a chimeric PIV based on recombinant engineering of infectious PIV clones. A variety of alterations can be produced in an isolated polynucleotide sequence encoding a targeted gene or genome segment, including a donor or recipient gene or genome segment in a chimeric PIV genome or antigenome for incorporation into infectious clones. More specifically, to achieve desired structural and phenotypic changes in recombinant PIV, the invention allows for introduction of modifications which delete, substitute, introduce, or rearrange a selected nucleotide or nucleotide sequence from a parent genome or antigenome, as well as mutations which delete, substitute, introduce or rearrange whole gene(s) or genome segment(s), within a chimeric PIV clone.

Thus provided are modifications in chimeric PIV of the invention which simply alter or ablate expression of a selected gene, e.g., by introducing a termination codon within a selected PIV coding sequence or altering its translational start site or RNA editing site, changing the position of a PIV gene relative to an operably linked promoter, introducing an upstream start codon to alter rates of expression, modifying (e.g., by changing position, altering an existing sequence, or substituting an existing sequence with a heterologous sequence) GS and/or GE transcription signals to alter phenotype (e.g., growth, temperature restrictions on transcription, etc.), and various other deletions, substitutions, additions and rearrangements that specify quantitative or qualitative changes in viral replication, transcription of selected gene(s), or translation of selected protein(s). In this context, any PIV gene or genome segment which is not essential for growth can be ablated or otherwise modified in a recombinant PIV to yield desired effects on virulence, pathogenesis, immunogenicity and other phenotypic characters. As for coding sequences, noncoding, leader, trailer and intergenic regions can be similarly deleted, substituted or modified and their phenotypic effects readily analyzed, e.g., by the use of minireplicons and recombinant PIV.

In addition, a variety of other genetic alterations can be produced in a PIV genome or antigenome for incorporation into a chimeric PIV, alone or together with one or more attenuating mutations adopted from a biologically derived mutant PIV, e.g., to adjust growth, attenuation, immunogenicity, genetic stability or provide other advantageous structural

and/or phenotypic effects. These additional types of mutations are also disclosed in the foregoing incorporated references and can be readily engineered into chimeric PIV of the invention. For example, restriction site markers are routinely introduced within chimeric PIVs to facilitate cDNA construction and manipulation.

5 In addition to these changes, the order of genes in a chimeric PIV construct can be changed, a PIV genome promoter replaced with its antigenome counterpart, portions of genes removed or substituted, and even entire genes deleted. Different or additional modifications in the sequence can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions or elsewhere. Nontranslated gene
10 sequences can be removed to increase capacity for inserting foreign sequences.

Other mutations for incorporation into chimeric PIV constructs of the invention include mutations directed toward cis-acting signals, which can be readily identified, e.g., by mutational analysis of PIV minigenomes. For example, insertional and deletional analysis of the leader and trailer and flanking sequences identifies viral promoters and transcription signals
15 and provides a series of mutations associated with varying degrees of reduction of RNA replication or transcription. Saturation mutagenesis (whereby each position in turn is modified to each of the nucleotide alternatives) of these cis-acting signals also has identified many mutations which affect RNA replication or transcription. Any of these mutations can be inserted into a chimeric PIV antigenome or genome as described herein. Evaluation and
20 manipulation of trans-acting proteins and cis-acting RNA sequences using the complete antigenome cDNA is assisted by the use of PIV minigenomes as described in the above-incorporated references.

Additional mutations within chimeric PIVs of the invention may also include replacement of the 3' end of genome with its counterpart from antigenome, which is associated
25 with changes in RNA replication and transcription. In one exemplary embodiment, the level of expression of specific PIV proteins, such as the protective HN and/or F antigens, can be increased by substituting the natural sequences with ones which have been made synthetically and designed to be consistent with efficient translation. In this context, it has been shown that codon usage can be a major factor in the level of translation of mammalian viral proteins (Haas
30 et al., Current Biol. 6:315-324, 1996, incorporated herein by reference). Optimization by recombinant methods of the codon usage of the mRNAs encoding the HN and F proteins of PIV will provide improved expression for these genes.

In another exemplary embodiment, a sequence surrounding a translational start site (preferably including a nucleotide in the -3 position) of a selected PIV gene is modified, alone or in combination with introduction of an upstream start codon, to modulate PIV gene expression by specifying up- or down-regulation of translation. Alternatively, or in combination with other recombinant modifications disclosed herein, gene expression of a chimeric PIV can be modulated by altering a transcriptional GS or GE signal of any selected gene(s) of the virus. In alternative embodiments, levels of gene expression in a chimeric PIV vaccine candidate are modified at the level of transcription. In one aspect, the position of a selected gene in the PIV gene map can be changed to a more promoter-proximal or promoter-distal position, whereby the gene will be expressed more or less efficiently, respectively. According to this aspect, modulation of expression for specific genes can be achieved yielding reductions or increases of gene expression from two-fold, more typically four-fold, up to ten-fold or more compared to wild-type levels often attended by a commensurate decrease in expression levels for reciprocally, positionally substituted genes. These and other transpositioning changes yield novel chimeric PIV vector virus having attenuated phenotypes, for example due to decreased expression of selected viral proteins involved in RNA replication, or having other desirable properties such as increased antigen expression.

In other embodiments, chimeric PIVs useful in vaccine formulations can be conveniently modified to accommodate antigenic drift in circulating virus. Typically the modification will be in the HN and/or F proteins. An entire HN or F gene, or a genome segment encoding a particular immunogenic region thereof, from one PIV strain or group is incorporated into a chimeric PIV genome or antigenome cDNA by replacement of a corresponding region in a recipient clone of a different PIV strain or group, or by adding one or more copies of the gene, such that multiple antigenic forms are represented. Progeny virus produced from the modified PIV clone can then be used in vaccination protocols against emerging PIV strains.

Replacement of a human PIV coding sequence or non-coding sequence (e.g., a promoter, gene-end, gene-start, intergenic or other cis-acting element) with a heterologous counterpart yields chimeric PIV having a variety of possible attenuating and other phenotypic effects. In particular, host range and other desired effects arise from substituting a bovine PIV (BPIV) or murine PIV (MPIV) protein, protein domain, gene or genome segment imported within a human PIV background, wherein the bovine or murine gene does not function efficiently in a human cell, e.g., from incompatibility of the heterologous sequence or protein

with a biologically interactive human PIV sequence or protein (i.e., a sequence or protein that ordinarily cooperates with the substituted sequence or protein for viral transcription, translation, assembly, etc.) or, more typically in a host range restriction, with a cellular protein or some other aspect of the cellular milieu which is different between the permissive and less permissive host. In exemplary embodiments, bovine PIV sequences are selected for introduction into human PIV based on known aspects of bovine and human PIV structure and function.

In more detailed aspects, the invention provides methods for attenuating chimeric PIV vaccine candidates based on the further construction of chimeras between HPIV and a non-human PIV, for example HPIV3 and BPIV3 (e.g., as disclosed in United States Provisional Application Serial No. 60/143,134 filed on July 9, 1999, incorporated herein by reference). This method of attenuation is based on host range effects due to the introduction of one or more gene(s) or genome segment(s) of the non-human PIV into a human PIV vector-based chimeric virus. For example, there are numerous nucleotide and amino acid sequence differences between BPIV and HPIVs, which are reflected in host range differences. Between HPIV3 and BPIV3 the percent amino acid identity for each of the following proteins is: N (86%), P (65%), M (93%), F (83%), HN (77%), and L (91%). The host range difference is exemplified by the highly permissive growth of HPIV3 in rhesus monkeys, compared to the restricted replication of two different strains of BPIV3 in the same animal (van Wyke Coelingh et al., J. Infect. Dis. 157:655-662, 1988, incorporated herein by reference). Although the basis of the host range differences between HPIV3 and BPIV3 remains to be determined, it is likely that they will involve more than one gene and multiple amino acid differences. The involvement of multiple genes and possibly cis-acting regulatory sequences, each involving multiple amino acid or nucleotide differences, gives a very broad basis for attenuation, one which cannot readily be altered by reversion. This is in contrast to the situation with other live attenuated HPIV3 viruses which are attenuated by one or several point mutations. In this case, reversion of any individual mutation may yield a significant reacquisition of virulence or, in a case where only a single residue specified attenuation, complete reacquisition of virulence.

In exemplary embodiments of the invention, the vector genome or antigenome is an HPIV3 genome or antigenome, and the heterologous gene or genome segment is a N ORF derived from, alternatively, a Ka or SF strain of BPIV3 (which are 99% related in amino acid sequence). The N ORF of the HPIV3 background antigenome is substituted by the counterpart BPIV3 N ORF—yielding a novel recombinant chimeric PIV clone. Replacement of the

HPIV3 N ORF of HPIV3 with that of BPIV3 Ka or SF results in a protein with approximately 70 amino acid differences (depending on the strain involved) from that of HPIV3 N. N is one of the more conserved proteins, and substitution of other proteins such as P, singly or in combination, would result in many more amino acid differences. The involvement of multiple
5 genes and genome segments each conferring multiple amino acid or nucleotide differences provides a broad basis for attenuation which is highly stable to reversion.

This mode of attenuation contrasts sharply to HPIV vaccine candidates that are attenuated by one or more point mutations, where reversion of an individual mutation may yield a significant or complete reacquisition of virulence. In addition, several known
10 attenuating point mutations in HPIV typically yield a temperature sensitive phenotype. One problem with attenuation associated with temperature sensitivity is that the virus can be overly restricted for replication in the lower respiratory tract while being under attenuated in the upper respiratory tract. This is because there is a temperature gradient within the respiratory tract, with temperature being higher (and more restrictive) in the lower respiratory tract and lower
15 (less restrictive) in the upper respiratory tract. The ability of an attenuated virus to replicate in the upper respiratory tract can result in complications including congestion, rhinitis, fever and otitis media. Thus, attenuation achieved solely by temperature sensitive mutations may not be ideal. In contrast, host range mutations present in chimeric PIV of the invention will not in most cases confer temperature sensitivity. Therefore, the novel method of PIV attenuation
20 provided by these kinds of modifications will be more stable genetically and phenotypically and less likely to be associated with residual virulence in the upper respiratory tract compared to other known PIV vaccine candidates.

The above-incorporated reference discloses that both Ka and SF HPIV3/BPIV3 chimeric recombinants are viable and replicate as efficiently in cell culture as either HPIV3 or
25 BPIV3 parent—indicating that the chimeric recombinants did not exhibit gene incompatibilities that restricted replication *in vitro*. This property of efficient replication *in vitro* is important since it permits efficient manufacture of this biological. Also, the Ka and the SF HPIV3/BPIV3 chimeric recombinants (termed cKa and cSF), bearing only one bovine gene, are nearly equivalent to their BPIV3 parents in the degree of host range restriction in the
30 respiratory tract of the rhesus monkey. In particular, the cKa and cSF viruses exhibit approximately a 60-fold or 30-fold reduction, respectively, in replication in the upper respiratory tract of rhesus monkeys compared to replication of HPIV3. Based on this finding, it is expected that other BPIV3 genes will also confer desired levels of host range restriction

within chimeric PIV of the invention. Thus, according to the methods herein, a list of attenuating determinants will be readily identified in heterologous genes and genome segments of BPIV and other non-human PIVs that will confer, in appropriate combination, a desired level of host range restriction and immunogenicity on chimeric PIV selected for vaccine use.

5 In preferred chimeric vaccine candidates of the invention, attenuation marked by replication in the lower and/or upper respiratory tract in an accepted animal model for PIV replication in humans, e.g., hamsters or rhesus monkeys, may be reduced by at least about two-fold, more often about 5-fold, 10-fold, or 20-fold, and preferably 50-100-fold and up to 1,000-fold or greater overall (e.g., as measured between 3-8 days following infection) compared to
10 growth of the corresponding wild-type or mutant parental PIV strain.

Infectious chimeric PIV vector clones of the invention can also be engineered according to the methods and compositions disclosed herein to enhance immunogenicity and induce a level of protection greater than that provided by infection with a wild-type, parental (i.e., vector or heterologous donor) PIV or non-PIV pathogen. For example, one or more
15 supplemental immunogenic epitope(s), protein domains, or proteins from a heterologous PIV strain or type, or from a non-PIV pathogen such as measles or RSV, can be added to a chimeric PIV by appropriate nucleotide changes in the chimeric genome or antigenome. Alternatively, chimeric PIVs of the invention can be engineered to add or ablate (e.g., by amino acid insertion, substitution or deletion) immunogenic proteins, protein domains, or forms of specific
20 proteins associated with desirable or undesirable immunological reactions.

Within the methods of the invention, additional genes or genome segments may be inserted into or proximate to the chimeric PIV vector genome or antigenome. These genes may be under common control with recipient genes, or may be under the control of an independent set of transcription signals. In addition to genes and genome segments encoding
25 antigenic determinants, genes of interest in this context include genes encoding cytokines, for example, an interleukin (e.g., interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL6), interleukin 18 (IL-18)), tumor necrosis factor alpha (TNF α), interferon gamma (IFN γ), or granulocyte-macrophage colony stimulating factor (GM-CSF), as well as IL-2 through IL-18, especially IL-2, IL-6 and IL-12, and IL-18, gamma-interferon (see, e.g.,
30 United States Provisional Application Serial No. 60/143,425 filed July 13, 1999, incorporated herein by reference). Coexpression of these additional proteins provides the ability to modify

and improve immune responses against chimeric PIV of the invention both quantitatively and qualitatively.

Deletions, insertions, substitutions and other mutations involving changes of whole viral genes or genome segments within chimeric PIV of the invention yield highly stable vaccine candidates, which are particularly important in the case of immunosuppressed individuals. Many of these changes will result in attenuation of resultant vaccine strains, whereas others will specify different types of desired phenotypic changes. For example, accessory (i.e., not essential for *in vitro* growth) genes are excellent candidates to encode proteins that specifically interfere with host immunity (see, e.g., Kato et al., EMBO. J. 16:578-87, 1997, incorporated herein by reference). Ablation of such genes in vaccine viruses is expected to reduce virulence and pathogenesis and/or improve immunogenicity.

Introduction of the foregoing defined mutations into an infectious, chimeric PIV clone can be achieved by a variety of well known methods. By "infectious clone" with regard to DNA is meant cDNA or its product, synthetic or otherwise, which can be transcribed into genomic or antigenomic RNA capable of serving as template to produce the genome of an infectious virus or subviral particle. Thus, defined mutations can be introduced by conventional techniques (e.g., site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of antigenome or genome cDNA subfragments to assemble a complete antigenome or genome cDNA as described herein has the advantage that each region can be manipulated separately (smaller cDNAs are easier to manipulate than large ones) and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or any subfragment thereof, can be used as template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using the Muta-gene® kit of Bio-Rad Laboratories (Richmond, CA) or a method using a double-stranded plasmid directly as template such as the Chameleon mutagenesis kit of Stratagene (La Jolla, CA), or by the polymerase chain reaction employing either an oligonucleotide primer or template which contains the mutation(s) of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis techniques are known and available for use in producing the mutations of interest in the PIV antigenome or genome cDNA. Mutations can vary from single nucleotide changes to replacement of large cDNA pieces containing one or more genes or genome regions.

Thus, in one illustrative embodiment mutations are introduced by using the Muta-gene phagemid *in vitro* mutagenesis kit available from Bio-Rad. In brief, cDNA encoding a portion of a PIV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies, Gaithersburg, MD). Phagemid preparations are prepared as recommended by the manufacturer. Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome fragment is then amplified and the mutated piece is then reintroduced into the full-length genome or antigenome clone.

The invention also provides methods for producing infectious chimeric PIV from one or more isolated polynucleotides, e.g., one or more cDNAs. According to the present invention cDNA encoding a PIV genome or antigenome is constructed for intracellular or *in vitro* coexpression with the necessary viral proteins to form infectious PIV. By "PIV antigenome" is meant an isolated positive-sense polynucleotide molecule which serves as the template for the synthesis of progeny PIV genome. Preferably a cDNA is constructed which is a positive-sense version of the PIV genome, corresponding to the replicative intermediate RNA, or antigenome, so as to minimize the possibility of hybridizing with positive-sense transcripts of the complementing sequences that encode proteins necessary to generate a transcribing, replicating nucleocapsid, i.e., sequences that encode N, P, and L proteins.

For purposes of the present invention the genome or antigenome of the recombinant PIV of the invention need only contain those genes or portions thereof necessary to render the viral or subviral particles encoded thereby infectious. Further, the genes or portions thereof may be provided by more than one polynucleotide molecule, i.e., a gene may be provided by complementation or the like from a separate nucleotide molecule, or can be expressed directly from the genome or antigenome cDNA.

By recombinant PIV is meant a PIV or PIV-like viral or subviral particle derived directly or indirectly from a recombinant expression system or propagated from virus or subviral particles produced therefrom. The recombinant expression system will employ a recombinant expression vector which comprises an operably linked transcriptional unit comprising an assembly of at least a genetic element or elements having a regulatory role in PIV gene expression, for example, a promoter, a structural or coding sequence which is transcribed into PIV RNA, and appropriate transcription initiation and termination sequences.

To produce infectious PIV from cDNA-expressed genome or antigenome, the genome or antigenome is coexpressed with those PIV proteins necessary to (i) produce a nucleocapsid capable of RNA replication, and (ii) render progeny nucleocapsids competent for both RNA replication and transcription. Transcription by the genome nucleocapsid provides the other PIV proteins and initiates a productive infection. Alternatively, additional PIV proteins needed for a productive infection can be supplied by coexpression.

Infectious PIV of the invention are produced by intracellular or cell-free coexpression of one or more isolated polynucleotide molecules that encode a PIV genome or antigenome RNA, together with one or more polynucleotides encoding viral proteins necessary to generate a transcribing, replicating nucleocapsid. Among the viral proteins useful for coexpression to yield infectious PIV are the major nucleocapsid protein (N) protein, nucleocapsid phosphoprotein (P), large (L) polymerase protein, fusion protein (F), hemagglutinin-neuraminidase glycoprotein (HN), and matrix (M) protein. Also useful in this context are products of the C, D and V ORFs of PIV.

cDNAs encoding a PIV genome or antigenome are constructed for intracellular or *in vitro* coexpression with the necessary viral proteins to form infectious PIV. By "PIV antigenome" is meant an isolated positive-sense polynucleotide molecule which serves as a template for synthesis of progeny PIV genome. Preferably a cDNA is constructed which is a positive-sense version of the PIV genome corresponding to the replicative intermediate RNA, or antigenome, so as to minimize the possibility of hybridizing with positive-sense transcripts of complementing sequences encoding proteins necessary to generate a transcribing, replicating nucleocapsid.

In some embodiments of the invention the genome or antigenome of a recombinant PIV (rPIV) need only contain those genes or portions thereof necessary to render the viral or subviral particles encoded thereby infectious. Further, the genes or portions thereof may be provided by more than one polynucleotide molecule, i.e., a gene may be provided by complementation or the like from a separate nucleotide molecule. In other embodiments, the PIV genome or antigenome encodes all functions necessary for viral growth, replication, and infection without the participation of a helper virus or viral function provided by a plasmid or helper cell line.

By "recombinant PIV" is meant a PIV or PIV-like viral or subviral particle derived directly or indirectly from a recombinant expression system or propagated from virus

or subviral particles produced therefrom. The recombinant expression system will employ a recombinant expression vector which comprises an operably linked transcriptional unit comprising an assembly of at least a genetic element or elements having a regulatory role in PIV gene expression, for example, a promoter, a structural or coding sequence which is transcribed into PIV RNA, and appropriate transcription initiation and termination sequences.

To produce infectious PIV from a cDNA-expressed PIV genome or antigenome, the genome or antigenome is coexpressed with those PIV N, P and L proteins necessary to (i) produce a nucleocapsid capable of RNA replication, and (ii) render progeny nucleocapsids competent for both RNA replication and transcription. Transcription by the genome nucleocapsid provides the other PIV proteins and initiates a productive infection. Alternatively, additional PIV proteins needed for a productive infection can be supplied by coexpression.

Synthesis of PIV antigenome or genome together with the above-mentioned viral proteins can also be achieved *in vitro* (cell-free), e.g., using a combined transcription-translation reaction, followed by transfection into cells. Alternatively, antigenome or genome RNA can be synthesized *in vitro* and transfected into cells expressing PIV proteins.

In certain embodiments of the invention, complementing sequences encoding proteins necessary to generate a transcribing, replicating PIV nucleocapsid are provided by one or more helper viruses. Such helper viruses can be wild type or mutant. Preferably, the helper virus can be distinguished phenotypically from the virus encoded by the PIV cDNA. For example, it is desirable to provide monoclonal antibodies which react immunologically with the helper virus but not the virus encoded by the PIV cDNA. Such antibodies can be neutralizing antibodies. In some embodiments, the antibodies can be used in affinity chromatography to separate the helper virus from the recombinant virus. To aid the procurement of such antibodies, mutations can be introduced into the PIV cDNA to provide antigenic diversity from the helper virus, such as in the HN or F glycoprotein genes.

In alternate embodiments of the invention, the N, P, L and other desired PIV proteins are encoded by one or more non-viral expression vectors, which can be the same or separate from that which encodes the genome or antigenome. Additional proteins may be included as desired, each encoded by its own vector or by a vector encoding one or more of the N, P, L and other desired PIV proteins, or the complete genome or antigenome. Expression of the genome or antigenome and proteins from transfected plasmids can be achieved, for

example, by each cDNA being under the control of a promoter for T7 RNA polymerase, which in turn is supplied by infection, transfection or transduction with an expression system for the T7 RNA polymerase, e.g., a vaccinia virus MVA strain recombinant which expresses the T7 RNA polymerase (Wyatt et al., Virology, 210: 202-205 (1995), incorporated herein by reference in its entirety). The viral proteins, and/or T7 RNA polymerase, can also be provided by transformed mammalian cells or by transfection of preformed mRNA or protein.

A PIV antigenome may be constructed for use in the present invention by, e.g., assembling cloned cDNA segments, representing in aggregate the complete antigenome, by polymerase chain reaction or the like (PCR; described in, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202, and *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, San Diego (1990), each incorporated herein by reference in its entirety) of reverse-transcribed copies of PIV mRNA or genome RNA. For example, a first construct is generated which comprises cDNAs containing the left hand end of the antigenome, spanning from an appropriate promoter (e.g., T7 RNA polymerase promoter) and assembled in an appropriate expression vector, such as a plasmid, cosmid, phage, or DNA virus vector. The vector may be modified by mutagenesis and/or insertion of synthetic polylinker containing unique restriction sites designed to facilitate assembly. For ease of preparation the N, P, L and other desired PIV proteins can be assembled in one or more separate vectors. The right hand end of the antigenome plasmid may contain additional sequences as desired, such as a flanking ribozyme and tandem T7 transcriptional terminators. The ribozyme can be hammerhead type (e.g., Grosfeld et al., (1995), *supra*), which would yield a 3' end containing a single nonviral nucleotide, or can be any of the other suitable ribozymes such as that of hepatitis delta virus (Perrotta et al., Nature 350:434-436, 1991), incorporated herein by reference in its entirety) which would yield a 3' end free of non-PIV nucleotides. The left- and right-hand ends are then joined via a common restriction site.

A variety of nucleotide insertions, deletions and rearrangements can be made in the PIV genome or antigenome during or after construction of the cDNA. For example, specific desired nucleotide sequences can be synthesized and inserted at appropriate regions in the cDNA using convenient restriction enzyme sites. Alternatively, such techniques as site-specific mutagenesis, alanine scanning, PCR mutagenesis, or other such techniques well known in the art can be used to introduce mutations into the cDNA.

Alternative means to construct cDNA encoding the genome or antigenome include reverse transcription-PCR using improved PCR conditions (e.g., as described in Cheng et al., Proc. Natl. Acad. Sci. USA 91:5695-5699 (1994)), incorporated herein by reference) to reduce the number of subunit cDNA components to as few as one or two pieces. In other
5 embodiments different promoters can be used (e.g., T3, SP6) or different ribozymes (e.g., that of hepatitis delta virus. Different DNA vectors (e.g., cosmids) can be used for propagation to better accommodate the larger size genome or antigenome.

Isolated polynucleotides (e.g., cDNA) encoding the genome or antigenome may be inserted into appropriate host cells by transfection, electroporation, mechanical insertion,
10 transduction or the like, into cells which are capable of supporting a productive PIV infection, e.g., HEp-2, FRhL-DBS2, LLC-MK2, MRC-5, and Vero cells. Transfection of isolated polynucleotide sequences may be introduced into cultured cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14: 725 (1978); Corsaro and Pearson, Somatic Cell Genetics 7: 603 (1981); Graham and Van der Eb, Virology 52: 456 (1973)),
15 electroporation (Neumann et al., EMBO J. 1: 841-845 (1982)), DEAE-dextran mediated transfection (Ausubel et al., (ed.) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY (1987), cationic lipid-mediated transfection (Hawley-Nelson et al., Focus 15: 73-79 (1993)) or a commercially available transfection reagent, e.g., LipofectACE® (Life Technologies) or the like (each of the foregoing references are incorporated herein by
20 reference in its entirety).

As noted above, in some embodiments of the invention the N, P, L and other desired PIV proteins are encoded by one or more helper viruses which is phenotypically distinguishable from that which encodes the genome or antigenome. The N, P, L and other desired PIV proteins can also be encoded by one or more expression vectors which can be the
25 same or separate from that which encodes the genome or antigenome, and various combinations thereof. Additional proteins may be included as desired, encoded by its own vector or by a vector encoding one or more of the N, P, L and other desired PIV proteins, or the complete genome or antigenome.

By providing infectious clones of PIV the invention permits a wide range of
30 alterations to be recombinantly produced within the PIV genome (or antigenome), yielding defined mutations which specify desired phenotypic changes. By "infectious clone" is meant cDNA or its product, synthetic or otherwise, RNA capable of being directly incorporated into

infectious virions which can be transcribed into genomic or antigenomic RNA capable of serving as a template to produce the genome of infectious viral or subviral particles. As noted above, defined mutations can be introduced by a variety of conventional techniques (e.g., site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of genomic or antigenomic cDNA subfragments to assemble a complete genome or antigenome cDNA as described herein has the advantage that each region can be manipulated separately, where small cDNA subjects provide for better ease of manipulation than large cDNA subjects, and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or a selected subfragment thereof, can be used as a template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using the MUTA-gen® kit of Bio-Rad Laboratories (Richmond, CA), or a method using the double-stranded plasmid directly as a template such as the Chameleon® mutagenesis kit of Strategene (La Jolla, CA), or by the polymerase chain reaction employing either an oligonucleotide primer or a template which contains the mutation(s) of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis techniques are known and can be routinely adapted for use in producing the mutations of interest in a PIV antigenome or genome cDNA of the invention.

Thus, in one illustrative embodiment mutations are introduced by using the MUTA-gene® phagemid *in vitro* mutagenesis kit available from Bio-Rad Laboratories. In brief, cDNA encoding an PIV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies). Phagemid preparations are prepared as recommended by the manufacturer. Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome is then amplified.

Mutations can vary from single nucleotide changes to the introduction, deletion or replacement of large cDNA segments containing one or more genes or genome segments. Genome segments can correspond to structural and/or functional domains, e.g., cytoplasmic, transmembrane or ectodomains of proteins, active sites such as sites that mediate binding or other biochemical interactions with different proteins, epitopic sites, e.g., sites that stimulate antibody binding and/or humoral or cell mediated immune responses, etc. Useful genome segments in this regard range from about 15-35 nucleotides in the case of genome segments encoding small functional domains of proteins, e.g., epitopic sites, to about 50, 75, 100, 200-500, and 500-1,500 or more nucleotides.

The ability to introduce defined mutations into infectious PIV has many applications, including the manipulation of PIV pathogenic and immunogenic mechanisms. For example, the functions of PIV proteins, including the N, P, M, F, HN, and L proteins and C, D and V ORF products, can be manipulated by introducing mutations which ablate or
5 reduce the level of protein expression, or which yield mutant protein. Various genome RNA structural features, such as promoters, intergenic regions, and transcription signals, can also be routinely manipulated within the methods and compositions of the invention. The effects of trans-acting proteins and cis-acting RNA sequences can be readily determined, for example, using a complete antigenome cDNA in parallel assays employing PIV minigenomes (Dimock,
10 et al., J. Virol. 67: 2772-8 (1993), incorporated herein by reference in its entirety), whose rescue-dependent status is useful in characterizing those mutants that may be too inhibitory to be recovered in replication-independent infectious virus.

Certain substitutions, insertions, deletions or rearrangements of genes or genome segments within recombinant PIV of the invention (e.g., substitutions of a genome
15 segment encoding a selected protein or protein region, for instance a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc.) are made in structural or functional relation to an existing, "counterpart" gene or genome segment from the same or different PIV or other source. Such modifications yield novel recombinants having desired phenotypic changes
20 compared to wild-type or parental PIV or other viral strains. For example, recombinants of this type may express a chimeric protein having a cytoplasmic tail and/or transmembrane domain of one PIV fused to an ectodomain of another PIV. Other exemplary recombinants of this type express duplicate protein regions, such as duplicate immunogenic regions.

As used herein, "counterpart" genes, genome segments, proteins or protein
25 regions, are typically from heterologous sources (e.g., from different PIV genes, or representing the same (i.e., homologous or allelic) gene or genome segment in different PIV types or strains). Typical counterparts selected in this context share gross structural features, e.g., each counterpart may encode a comparable protein or protein structural domain, such as a cytoplasmic domain, transmembrane domain, ectodomain, binding site or region, epitopic site
30 or region, etc. Counterpart domains and their encoding genome segments embrace an assemblage of species having a range of size and sequence variations defined by a common biological activity among the domain or genome segment variants.

Counterpart genes and genome segments, as well as other polynucleotides disclosed herein for producing recombinant PIV within the invention, often share substantial sequence identity with a selected polynucleotide "reference sequence," e.g., with another selected counterpart sequence. As used herein, a "reference sequence" is a defined sequence used as a basis for sequence comparison, for example, a segment of a full-length cDNA or gene, or a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. USA 85:2444 (1988) (each of which is incorporated by reference), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI, incorporated herein by reference), or by inspection, and the best alignment (i.e., resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and

5 multiplying the result by 100 to yield the percentage of sequence identity. The terms
"substantial identity" as used herein denotes a characteristic of a polynucleotide sequence,
wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity,
preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence
10 identity as compared to a reference sequence over a comparison window of at least 20
nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the
percentage of sequence identity is calculated by comparing the reference sequence to the
polynucleotide sequence which may include deletions or additions which total 20 percent or
less of the reference sequence over the window of comparison. The reference sequence may
15 be a subset of a larger sequence.

In addition to these polynucleotide sequence relationships, proteins and protein
regions encoded by recombinant PIV of the invention are also typically selected to have
conservative relationships, i.e. to have substantial sequence identity or sequence similarity,
with selected reference polypeptides. As applied to polypeptides, the term "sequence identity"
15 means peptides share identical amino acids at corresponding positions. The term "sequence
similarity" means peptides have identical or similar amino acids (i.e., conservative
substitutions) at corresponding positions. The term "substantial sequence identity" means that
two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT
using default gap weights, share at least 80 percent sequence identity, preferably at least 90
20 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g.,
99 percent sequence identity). The term "substantial similarity" means that two peptide
sequences share corresponding percentages of sequence similarity. Preferably, residue
positions which are not identical differ by conservative amino acid substitutions. Conservative
amino acid substitutions refer to the interchangeability of residues having similar side chains.
25 For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine,
leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine
and threonine; a group of amino acids having amide-containing side chains is asparagine and
glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and
tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine;
30 and a group of amino acids having sulfur-containing side chains is cysteine and methionine.
Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine,
phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

Abbreviations for the twenty naturally occurring amino acids used herein follow conventional
usage (Immunology - A Synthesis (2nd ed., E.S. Golub & D.R. Gren, eds., Sinauer Associates,

Sunderland, MA, 1991), incorporated herein by reference). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α,α -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω -N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). Moreover, amino acids may be modified by glycosylation, phosphorylation and the like.

To select candidate vaccine viruses according to the invention, the criteria of viability, attenuation and immunogenicity are determined according to well known methods. Viruses which will be most desired in vaccines of the invention must maintain viability, have a stable attenuation phenotype, exhibit replication in an immunized host (albeit at lower levels), and effectively elicit production of an immune response in a vaccinee sufficient to confer protection against serious disease caused by subsequent infection from wild-type virus. The recombinant PIV of the invention are not only viable and more appropriately attenuated than previous vaccine candidates, but are more stable genetically *in vivo*--retaining the ability to stimulate a protective immune response and in some instances to expand the protection afforded by multiple modifications, e.g., induce protection against different viral strains or subgroups, or protection by a different immunologic basis, e.g., secretory versus serum immunoglobulins, cellular immunity, and the like.

Recombinant PIV of the invention can be tested in various well known and generally accepted *in vitro* and *in vivo* models to confirm adequate attenuation, resistance to phenotypic reversion, and immunogenicity for vaccine use. In *in vitro* assays, the modified virus (e.g., a multiply attenuated, biologically derived or recombinant PIV) is tested, e.g., for temperature sensitivity of virus replication, i.e. its phenotype, and for the small plaque or other desired phenotype. Modified viruses are further tested in animal models of PIV infection. A variety of animal models have been described and are summarized in various references incorporated herein. PIV model systems, including rodents and non-human primates, for evaluating attenuation and immunogenic activity of PIV vaccine candidates are widely accepted in the art, and the data obtained therefrom correlate well with PIV infection, attenuation and immunogenicity in humans.

In accordance with the foregoing description, the invention also provides isolated, infectious recombinant PIV compositions for vaccine use. The attenuated virus which is a component of a vaccine is in an isolated and typically purified form. By isolated is meant to refer to PIV which is in other than a native environment of a wild-type virus, such as the nasopharynx of an infected individual. More generally, isolated is meant to include the attenuated virus as a component of a cell culture or other artificial medium where it can be propagated and characterized in a controlled setting. For example, attenuated PIV of the invention may be produced by an infected cell culture, separated from the cell culture and added to a stabilizer.

For vaccine use, recombinant PIV produced according to the present invention can be used directly in vaccine formulations, or lyophilized, as desired, using lyophilization protocols well known to the artisan. Lyophilized virus will typically be maintained at about 4°C. When ready for use the lyophilized virus is reconstituted in a stabilizing solution, e.g., saline or comprising SPG, Mg^{++} and HEPES, with or without adjuvant, as further described below.

PIV vaccines of the invention contain as an active ingredient an immunogenically effective amount of PIV produced as described herein. The modified virus may be introduced into a host with a physiologically acceptable carrier and/or adjuvant. Useful carriers are well known in the art, and include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and the like. Acceptable adjuvants include incomplete Freund's adjuvant, MPL™ (3-o-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, MT) and IL-12 (Genetics Institute, Cambridge MA), among many other suitable adjuvants well known in the art.

Upon immunization with a PIV composition as described herein, via aerosol, droplet, oral, topical or other route, the immune system of the host responds to the vaccine by producing antibodies specific for PIV proteins, e.g., F and HN glycoproteins. As a result of the

vaccination with an immunogenically effective amount of PIV produced as described herein, the host becomes at least partially or completely immune to PIV infection, or resistant to developing moderate or severe PIV infection, particularly of the lower respiratory tract.

The host to which the vaccines are administered can be any mammal which is susceptible to infection by PIV or a closely related virus and which host is capable of generating a protective immune response to the antigens of the vaccinating strain. Accordingly, the invention provides methods for creating vaccines for a variety of human and veterinary uses.

The vaccine compositions containing the PIV of the invention are administered to a host susceptible to or otherwise at risk for PIV infection to enhance the host's own immune response capabilities. Such an amount is defined to be a "immunogenically effective dose." In this use, the precise amount of PIV to be administered within an effective dose will depend on the host's state of health and weight, the mode of administration, the nature of the formulation, etc., but will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per host, more commonly from about 10^4 to 10^6 PFU virus per host. In any event, the vaccine formulations should provide a quantity of modified PIV of the invention sufficient to effectively protect the host patient against serious or life-threatening PIV infection.

The PIV produced in accordance with the present invention can be combined with viruses of other PIV serotypes or strains to achieve protection against multiple PIV serotypes or strains. Alternatively, protection against multiple PIV serotypes or strains can be achieved by combining protective epitopes of multiple serotypes or strains engineered into one virus, as described herein. Typically when different viruses are administered they will be in admixture and administered simultaneously, but they may also be administered separately. Immunization with one strain may protect against different strains of the same or different serotype.

In some instances it may be desirable to combine the PIV vaccines of the invention with vaccines which induce protective responses to other agents, particularly other childhood viruses. In another aspect of the invention the PIV can be employed as a vector for protective antigens of other pathogens, such as respiratory syncytial virus (RSV) or measles virus, by incorporating the sequences encoding those protective antigens into the PIV genome or antigenome which is used to produce infectious PIV, as described herein.

In all subjects, the precise amount of recombinant PIV vaccine administered, and the timing and repetition of administration, will be determined based on the patient's state of health and weight, the mode of administration, the nature of the formulation, etc. Dosages will generally range from about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per patient, more commonly from about 10^4 to 10^6 PFU virus per patient. In any event, the vaccine formulations should provide a quantity of attenuated PIV sufficient to effectively stimulate or induce an anti-PIV immune response, e.g., as can be determined by complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay, among other methods. In this regard, individuals are also monitored for signs and symptoms of upper respiratory illness. As with administration to chimpanzees, the attenuated virus of the vaccine grows in the nasopharynx of vaccinees at levels approximately 10-fold or more lower than wild-type virus, or approximately 10-fold or more lower when compared to levels of incompletely attenuated PIV.

In neonates and infants, multiple administration may be required to elicit sufficient levels of immunity. Administration should begin within the first month of life, and at intervals throughout childhood, such as at two months, six months, one year and two years, as necessary to maintain sufficient levels of protection against native (wild-type) PIV infection. Similarly, adults who are particularly susceptible to repeated or serious PIV infection, such as, for example, health care workers, day care workers, family members of young children, the elderly, individuals with compromised cardiopulmonary function, may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection. Further, different vaccine viruses may be indicated for administration to different recipient groups. For example, an engineered PIV strain expressing a cytokine or an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

PIV vaccines produced in accordance with the present invention can be combined with viruses expressing antigens of another subgroup or strain of PIV to achieve protection against multiple PIV subgroups or strains. Alternatively, the vaccine virus may incorporate protective epitopes of multiple PIV strains or subgroups engineered into one PIV clone, as described herein.

The PIV vaccines of the invention elicit production of an immune response that is protective against serious lower respiratory tract disease, such as pneumonia and bronchiolitis when the individual is subsequently infected with wild--type PIV. While the naturally circulating virus is still capable of causing infection, particularly in the upper respiratory tract, there is a very greatly reduced possibility of rhinitis as a result of the vaccination and possible boosting of resistance by subsequent infection by wild-type virus. Following vaccination, there are detectable levels of host engendered serum and secretory antibodies which are capable of neutralizing homologous (of the same subgroup) wild-type virus *in vitro* and *in vivo*. In many instances the host antibodies will also neutralize wild-type virus of a different, non-vaccine subgroup.

Preferred PIV vaccine candidates of the invention exhibit a very substantial diminution of virulence when compared to wild-type virus that is circulating naturally in humans. The virus is sufficiently attenuated so that symptoms of infection will not occur in most immunized individuals. In some instances the attenuated virus may still be capable of dissemination to unvaccinated individuals. However, its virulence is sufficiently abrogated such that severe lower respiratory tract infections in the vaccinated or incidental host do not occur.

The level of attenuation of PIV vaccine candidates may be determined by, for example, quantifying the amount of virus present in the respiratory tract of an immunized host and comparing the amount to that produced by wild-type PIV or other attenuated PIV which have been evaluated as candidate vaccine strains. For example, the attenuated virus of the invention will have a greater degree of restriction of replication in the upper respiratory tract of a highly susceptible host, such as a chimpanzee, compared to the levels of replication of wild-type virus, e.g., 10- to 1000-fold less. In order to further reduce the development of rhinorrhea, which is associated with the replication of virus in the upper respiratory tract, an ideal vaccine candidate virus should exhibit a restricted level of replication in both the upper and lower respiratory tract. However, the attenuated viruses of the invention must be sufficiently infectious and immunogenic in humans to confer protection in vaccinated individuals. Methods for determining levels of PIV in the nasopharynx of an infected host are well known in the literature.

Levels of induced immunity provided by the vaccines of the invention can also be monitored by measuring amounts of neutralizing secretory and serum antibodies. Based on

these measurements, vaccine dosages can be adjusted or vaccinations repeated as necessary to maintain desired levels of protection. Further, different vaccine viruses may be advantageous for different recipient groups. For example, an engineered PIV strain expressing an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

In yet another aspect of the invention the PIV is employed as a vector for transient gene therapy of the respiratory tract. According to this embodiment the recombinant PIV genome or antigenome incorporates a sequence which is capable of encoding a gene product of interest. The gene product of interest is under control of the same or a different promoter from that which controls PIV expression. The infectious PIV produced by coexpressing the recombinant PIV genome or antigenome with the N, P, L and other desired PIV proteins, and containing a sequence encoding the gene product of interest, is administered to a patient. Administration is typically by aerosol, nebulizer, or other topical application to the respiratory tract of the patient being treated. Recombinant PIV is administered in an amount sufficient to result in the expression of therapeutic or prophylactic levels of the desired gene product. Representative gene products which may be administered within this method are preferably suitable for transient expression, including, for example, interleukin-2, interleukin-4, gamma-interferon, GM-CSF, G-CSF, erythropoietin, and other cytokines, glucocerebrosidase, phenylalanine hydroxylase, cystic fibrosis transmembrane conductance regulator (CFTR), hypoxanthine-guanine phosphoribosyl transferase, cytotoxins, tumor suppressor genes, antisense RNAs, and vaccine antigens.

The following examples are provided by way of illustration, not limitation. These examples document construction of representative chimeric PIVs bearing one or more heterologous antigenic determinant(s) according to the above described methods. In one example, the HA gene of the measles virus is inserted as an extra gene into one of three gene junctions of a JS wild type or attenuated strain of HPIV3, namely, the N/P, P/M, or HN/L junction, and recombinant chimeric viruses were recovered. Insertion of the measles HA gene at three different positions in the HPIV3 genome illustrates the range of useful constructs for transferring antigenic determinants from foreign pathogens into PIV vectors. Further, it is expected that inserted gene units that are more 3'-leader proximal will be transcribed and expressed at higher levels than the same gene units located more distally, which will allow for

closer modulation of heterologous gene expression (Collins et al., 3rd ed. In "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds.), Vol. 1, pp. 1205-1243. Lippincott-Raven Publishers, Philadelphia, 1996).

5 The chimeric rHPIV bearing the measles virus HA insertion in a wild type rHPIV3 background replicated efficiently *in vitro* but was restricted in replication in hamsters compared to that of the rHPIV3 virus from which it was derived. Similarly, the recombinant chimeric HPIV3 bearing the measles virus HA insertion in an attenuated rHPIV3 background replicated *in vitro* and in hamsters to a level that was also slightly less than that of the
10 attenuated rHPIV3 $cp45L$ mutant virus from which it was derived. The amount of HA protein expressed by cells infected with the attenuated rHPIV3-measles virus HA recombinants with the HA gene in the N/P or P/M junction was very high and even exceeded that seen in cells infected with native measles virus. The level of replication of the rHPIV3 $cp45L$ with a measles virus HA insert in the N/P or P/M junction was 10-fold lower in the upper respiratory
15 tract of the hamster than that of the rHPIV3- $cp45L$ parent virus indicating that gene insertions can unexpectedly contribute to the attenuation of an HPIV3 vector. These results which identify a unique host range phenotype are unexpected.

 Importantly, infection of hamsters with each recombinant chimeric virus tested induced high levels of antibody to both HPIV3 and to measles virus. Animals immunized with
20 the attenuated recombinant chimeric HPIV3 carrying the HA insertion were highly resistant to replication of HPIV3 challenge virus. While the wild type measles virus does not replicate efficiently in hamsters and thus cannot be used in challenge study, the protective efficacy of the attenuated recombinant chimeric vaccine is readily apparent from the high levels of neutralizing antibody induced. These levels are associated with a high level of resistance to
25 measles in humans (Chen et al., J. Infect. Dis. 162:1036-42, 1990).

 It is further demonstrated in the examples that attenuated chimeric recombinant HPIV vectors, combining a backbone of HPIV3 and one or more antigenic determinants of HPIV1, can also be used as vectors to express additional foreign antigens (e.g., of HPIV2 or a non-PIV virus). This aspect of the invention takes advantage of the efficient growth and
30 excellent attenuation properties of the HPIV3 backbone to carry antigenic determinants of multiple heterologous pathogens, as exemplified by HPIV1 and HPIV2. The cDNA encoding rPIV3-1 (a non-attenuated recombinant bearing major antigens of HPIV1) or rPIV3-1 $cp45$ (an

attenuated recombinant bearing HPIV1 major antigens) was modified by the insertion of a gene unit containing the ORF of HPIV2 HN gene between the gene units containing the F and HN ORFs of HPIV1. The recombinant chimeric viruses, designated rPIV3-1.2HN and rPIV3-1cp45.2HN, were readily recovered and replicated efficiently in tissue culture. Each virus exhibited a level of temperature sensitivity of replication *in vitro* similar to that of its rPIV3-1 or rPIV3-1cp45 parent virus. The insertion of the PIV2 HN attenuated both the rPIV3-1 and rPIV3-cp45 viruses in hamsters, a finding similar to that observed with the insertion of the measles viruses HA into rJS and into rPIV3cp45. Infection of hamsters with these antigenic rPIV3-1 recombinants bearing the PIV2 HN gene insert induced serum antibody responses reactive against both HPIV1 and HPIV2.

Thus, it is possible to use an attenuated rHPIV3 or rHPIV3-1 vaccine candidate as a vector to infect the respiratory tract of susceptible hosts and thereby induce a vigorous antibody response to foreign protective antigens expressed from an extra gene unit, as well as against the HPIV vector itself. The presence of three antigenic serotypes of HPIV, which do not provide significant cross-protection, allows for more effective, sequential immunization of human infants with antigenically distinct variants of HPIV each bearing the same or different heterologous antigenic determinant(s), e.g., a protective antigen, antigenic domain or epitope of measles virus or of one or more different viral or microbial pathogens. Sequential immunization permits development of a primary immune response to the foreign protein, which is boosted during subsequent infections with a secondary, antigenically-distinct HPIV bearing one or more heterologous antigenic determinants, e.g., a protective antigen, antigenic domain or epitope of measles virus or of one or more different viral or microbial pathogens. In this way, the immunity induced to one HPIV vector can be circumvented by boosting with an antigenically distinct HPIV vector. In this context, successful immunization of animals that are immune to PIV3 has been achieved with attenuated PIV3-1 vaccine candidates, confirming the feasibility of sequential immunization with serotypically distinct PIV viruses even if these PIVs share proteins other than HN and F. (Tao et al., Vaccine 17:1100-8, 1999). In this study, the immunogenicity and efficacy of rPIV3-1.cp45L against PIV1 challenge was examined in hamsters with and without prior immunity to PIV3. rPIV3-1.cp45L efficiently infected hamsters previously infected with wild type or attenuated PIV3, but there was approximately a five-fold reduction in replication of rPIV3-1.cp45L virus in the PIV3-immune animals. However, rPIV3-1.cp45L immunization of PIV3-immune animals induced a vigorous serum antibody response to PIV1 and reduced replication of PIV1 challenge virus 1000-fold in the lower respiratory tract and 200-fold in the upper respiratory tract. These results demonstrate

that the recombinant chimeric rPIV3-1.*cp45L* candidate vaccine can induce immunity to PIV1 even in animals immune to PIV3. This establishes the feasibility of employing a sequential immunization schedule in which a recombinant chimeric rPIV3-1.*cp45L* or other PIV vaccine virus is given following a live attenuated PIV3 vaccine. since rPIV3-1.*cp45L* readily induced protective immunity against itself, it would also induce an effective immune response to any vectored protective antigen that it was carrying. Also, the PIVs and RSV have the unusual property of being able to reinfect the respiratory tract, although reinfections typically are not associated with serious disease. Thus, vector based vaccine constructs of the invention are useful to boost immune responses by a second, third or fourth administration of the same HPIV vector or by sequential use of different vectors.

In preferred sequential vaccination methods of the invention, it is desirable to sequentially immunize an infant with different PIV vectors each expressing the same heterologous antigenic determinant such as the measles virus HA. This sequential immunization permits the induction of the high titer of antibody to the heterologous protein that is characteristic of the secondary antibody response. In one embodiment, early infants (e.g. 2-4 month old infants) are immunized with an attenuated chimeric HPIV3 expressing a heterologous antigenic determinant, for example the measles virus HA protein, and also adapted to elicit an immune response against HPIV3. One exemplary vaccine candidate useful in this context is the *rcp45L*(HA P-M) recombinant. Subsequently, e.g., at four months of age the infant is again immunized but with a different, secondary PIV vector construct antigenically distinct from the first. An exemplary vaccine candidate in this context is the rPIV3-1 *cp45L* virus expressing the measles virus HA gene and HPIV1 antigenic determinants as functional, obligate glycoproteins of the vector. Following the first vaccination, the vaccinee will elicit a primary antibody response to both the PIV3 HN and F proteins and to the measles virus HA protein, but not to the PIV1 HN and F protein. Upon secondary immunization with the rPIV3-1 *cp45L* expressing the measles virus HA, the vaccinee will be readily infected with the vaccine because of the absence of antibody to the PIV1 HN and F proteins and will develop both a primary antibody response to the PIV1 HN and F protective antigens and a high titered secondary antibody response to the heterologous measles virus HA protein. A similar sequential immunization schedule can be developed where immunity is sequentially elicited against HPIV3 and then HPIV2 by one or more of the chimeric vaccine viruses disclosed herein, simultaneous with stimulation of an initial and then secondary, high titer protective response against measles or another non-PIV pathogen. This sequential immunization strategy, preferably employing different serotypes of PIV as primary and

secondary vectors, effectively circumvents immunity that is induced to the primary vector, a factor ultimately limiting the usefulness of vectors with only one serotype.

Further in accordance with this aspect of the invention, exemplary coordinate vaccination protocols may incorporate two, three, four and up to six or more separate chimeric HPIV vaccine viruses administered simultaneously (e.g., in a polyspecific vaccine mixture) in a primary vaccination step, e.g., at one, two or four months of age. For example, two or more and up to a full panel of HPIV-based vaccine viruses can be administered that separately express one or more antigenic determinants (i.e., whole antigens, immunogenic domains, or epitopes) selected from the G protein of RSV subgroup A, the F protein of RSV subgroup A, the G protein of RSV subgroup B, the F protein of RSV subgroup B, the HA protein of measles virus, and/or the F protein of measles virus. Coordinate booster administration of these same PIV3-based vaccine constructs can be repeated at two months of age. Subsequently, e.g., at four months of age, a separate panel of 2-6 or more antigenically distinct (referring to vector antigenic specificity) live attenuated HPIV-based vaccine viruses can be administered in a secondary vaccination step. For example, secondary vaccination may involve concurrent administration of a mixture or multiple formulations that contain(s) multiple HPIV3-1 vaccine constructs that collectively express RSV G from subgroup A, RSV F from subgroup A, RSV F from subgroup B, RSV G from subgroup B, measles virus HA, and/or measles virus F, or antigenic determinants from any combination of these proteins. This secondary immunization provides a boost in immunity to each of the heterologous RSV and measles virus proteins or antigenic determinant(s) thereof. At six months of age, a tertiary vaccination step involving administration of one-six or more separate live attenuated PIV3-2 vector-based vaccine recombinants can be coordinately administered that separately or collectively express RSV G from subgroup A, RSV F from subgroup A, RSV G from subgroup B, RSV F from subgroup B, measles virus HA, and/or measles virus F, or antigenic determinant(s) thereof. Optionally at this step in the vaccination protocol, rPIV3 and rPIV3-1 vaccines may be administered in booster formulations. In this way, the strong immunity characteristic of secondary antibody to PIV1, PIV2, PIV3, RSV A, RSV B, and measles viruses are all induced within the first six months of infancy. Such a coordinate/sequential immunization strategy, which is able to induce secondary antibody responses to multiple viral respiratory pathogens, provides a highly powerful and extremely flexible immunization regimen that is driven by the need to immunize against each of the three PIV viruses and other pathogens in early infancy.

As described herein above and further detailed in the illustrative examples that follow, the present invention provides six major advantages over previous attempts to immunize the young infant against measles virus or other microbial pathogens. First, the PIV recombinant vector into which the protective antigen or antigens of measles virus or of other microbial pathogens is inserted is an attenuated rPIV bearing one or more attenuating genetic elements that are known to attenuate virus for the respiratory tract of the very young human infant (Karron et al., Pediatr. Infect. Dis. J. 15:650-654, 1996; Karron et al., J. Infect. Dis. 171:1107-1114, 1995a; Karron et al., J. Infect. Dis. 172:1445-1450, 1995b). This extensive history of prior clinical evaluation and practice greatly facilitates evaluation of derivatives of these recombinants bearing foreign protective antigens in the very young human infant.

A second advantage satisfied by the invention is that the rPIV backbone carrying the HA or other protective antigen of a heterologous, non-PIV human pathogen will induce a dual protective immune response against (1) one or more PIVs, for which there are compelling independent needs for vaccines as indicated above, and (2) the measles virus or other microbial pathogen whose protective antigen is expressed by the vector. This contrasts with the VSV-measles virus HA recombinant described above which will induce immunity to only one human pathogen, i.e., the measles virus, and in which the immune response to the vector itself is at best irrelevant or is potentially disadvantageous. The coding sequences of the foreign genes inserted into various members of the *Mononegavirales* Order of viruses have remained intact in the genomes of the most of the recombinant viruses following multiple cycles of replication in tissue culture cells, indicating that members of this group of viruses are excellent candidates for use as vectors (Bukreyev et al., J. Virol. 70:6634-41, 1996; Schnell et al., Proc. Natl. Acad. Sci. U S A 93:11359-65, 1996a; Singh et al., J. Gen. Virol. 80:101-6; Yu et al., Genes Cells 2:457-66, 1997).

Another advantage provided by the invention is that use of a human pathogen backbone, for which there is a need for a vaccine, will favor the introduction of such a live attenuated virus vector into an already crowded early childhood immunization schedule.

In addition, immunization via the mucosal surface of the respiratory tract offers various advantages. A live attenuated PIV3 was shown to replicate in the respiratory tract of rhesus monkeys and to induce a protective immune response against itself in the presence of high quantities of maternally-acquired PIV3-specific serum antibodies. The ability of two candidate PIV3 vaccines to infect and to replicate efficiently in the upper respiratory tract of

the very young human infant who possess maternally-acquired antibodies has also been demonstrated (Karron et al., Pediatr. Infect. Dis. J. 15:650-654, 1996; Karron et al., J. Infect. Dis. 171:1107-1114, 1995a; Karron et al., J. Infect. Dis. 172:1445-1450, 1995b). This is in contrast to the currently licensed measles virus vaccine which is poorly infectious when administered to the upper respiratory tract of humans and which is highly sensitive to neutralization when administered parenterally to young children (Black et al., New Eng. J. Med. 263:165-169, 1960; Kok et al., Trans. R. Soc. Trop. Med. Hyg. 77:171-6, 1983; Simasathien et al., Vaccine 15:329-34, 1997). The replication of the HPIV vector in the respiratory tract will stimulate the production of both mucosal IgA and systemic immunity to the HPIV vector and to the expressed foreign antigen. Upon subsequent natural exposure to wild type virus, e.g., measles virus, the existence of vaccine-induced local and systemic immunity should serve to restrict its replication at both its portal of entry, i.e., the respiratory tract, as well as at systemic sites of replication.

Also, the presence of three antigenic serotypes of HPIV, each of which causes significant disease in humans and hence can serve simultaneously as vector and vaccine, presents a unique opportunity to sequentially immunize the infant with antigenically distinct variants of HPIV each bearing the same foreign protein. In this manner the sequential immunization will permit the development of a primary immune response to the foreign protein which can be boosted during subsequent infections with the antigenically distinct HPIV also bearing the same or a different foreign protein or proteins, i.e., the protective antigen of measles virus or of another microbial pathogen. In this regard, several attenuated PIVs have been identified herein as exemplary vectors for use in this format of sequential immunization, e.g., PIV3cp45; PIV3-1cp45; PIV3-1cp45L, PIV3-2CT; and PIV3-2TM. It is also likely that readministration of homologous HPIV vectors will also boost the response to both HPIV and the foreign antigen since the ability to cause multiple reinfections in humans is an unusual but characteristic attribute of the HPIVs (Collins et al., In "Fields Virology" (B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus, Eds.), Vol. 1, pp. 1205-1243. Lippincott-Raven Publishers, Philadelphia, 1996).

Yet another advantage is that the introduction of a gene unit into a PIV vector has several unexpected, but highly desirable effects, for the production of attenuated viruses. First, the insertion of gene units expressing the HA of measles virus or the HN of PIV2 each specify a host range phenotype on the PIV vector that has not been previously recognized, i.e., the resulting PIV vector replicates efficiently *in vitro* but is restricted in replication *in vivo* in

both the upper and lower respiratory tracts. These findings identify the insertion of a gene unit expressing a viral protective antigen as an attenuating factor for the PIV vector, a desirable property in live attenuated virus vaccines of the invention.

The ability of chimeric HPIVs of the invention bearing heterologous sequences to replicate efficiently *in vitro* demonstrates the feasibility for large scale production of vaccine. This is in contrast to the replication of some single-stranded, negative-sense RNA viruses which can be inhibited *in vitro* by the insertion of a foreign gene (Bukreyev et al., J. Virol. 70:6634-41, 1996).

EXAMPLE I

Construction of cDNAs Encoding a Chimeric HPIV3/Measles Virus-HA

Antigenome and Recovery of Infectious Virus

Full-length cDNA clones, p3/7(131)2G+, encoding the complete 15462 nucleotide antigenome of the JS PIV3 wt virus, and pFLCcp45L, which encodes the antigenome of the derivative of JS wt containing three *cp45*-specific temperature-sensitive mutations in the L ORF of PIV3, have been previously described (Durbin et al., Virology 235:323-332, 1997a; Skiadopoulos et al., J. Virol. 72:1762-8, 1998, each incorporated herein by reference). These clones were used as vectors for the insertion of the HA gene of measles virus to create both wildtype and attenuated HPIV3 chimeric constructs which express a heterologous antigenic determinant, exemplified by the HA protein, of measles virus. The size of each insert containing the HA gene of measles was a multiple of six such that the chimeric virus recovered from the cDNA would conform to the rule of six (Durbin et al., Virology 234:74-83, 1997b, incorporated herein by reference).

Construction of full-length chimeric HPIV3 cDNAs encoding the HA protein of measles virus in the N/P or P/M junctions.

The *Pml*I to *Bam*HI fragment of p3/7(131)2G+ (nt 1215-3903 of the PIV3 antigenome) was subcloned into the plasmid pUC119 {pUC119(*Pml*I-*Bam*HI)} which had been modified to include a *Pml*I site in the multiple cloning region. Two independent single-stranded mutagenesis reactions were performed on pUC119(*Pml*I-*Bam*HI) using Kunkel's method (Kunkel et al., Methods Enzymol. 154:367-382, 1987, incorporated herein by

reference); the first reaction introduced an *A**ff**II* site in the 3' (downstream)-noncoding region of the N gene by mutating the CTAAAT sequence at nts 1677-1682 of the antigenome to CTTAAG (p*A**ff**II* N-P), and the second, separate, reaction introduced an *A**ff**II* site in the 3'-noncoding region of the P gene by mutating the TCAATC sequence at nts 3693-3698 of the antigenome to CTTAAG (p*A**ff**II* P-M).

Sub. 95
~~The HA ORF of measles virus Edmonston strain was amplified from Edmonston wild type virus by reverse transcription polymerase chain reaction (RT-PCR). The nt sequence of the Edmonston wild type HA open reading frame (ORF) is in GenBank Accession # U03669, incorporated herein by reference (note that this sequence is the ORF only without the upstream 3 nts or the stop codon). Measles virus RNA was purified from clarified medium using TRIzol-LS (Life Technologies, Gaithersburg, MD) following the manufacturer's recommended procedure. RT-PCR was performed with the Advantage RT-for-PCR and Advantage-HF PCR kits (Clontech, Palo Alto, CA) following the recommended protocols. Primers were used to generate a PCR fragment spanning the entire ORF of the measles virus HA gene flanked by PIV3 non-coding sequence and *A**ff**II* restriction sites. The forward primer 5'-~~
~~TTAATCTTAAGAATATACAAATAAGAAAACTTAGGATTAAAGAGCGATGTCACC~~
~~ACAACGAGACCGGATAAATGCCTTCTAC-3' encodes an *A**ff**II* site (italicized)~~
~~upstream of PIV3 noncoding sequence derived from the N/P gene junction-nts 3699-~~
~~3731(underlined), containing GE, IG and GS sequences (Figure 1A) and the beginning of the~~
~~measles HA ORF (bolded) preceded by three non-HPIV3, non-measles virus nts designated in~~
~~the primer. The reverse primer 5'-~~
~~ATTATTGCTTAAGGTTTGTTCGGTGTTCGTTTCTTTGTTGGATCCTATCTGCGATTGG~~
~~TTCCATCTTC-3' encodes an *A**ff**II* site (italicized) downstream (in the positive-sense~~
~~complement) of PIV3 noncoding sequence derived from the P gene, nt 3594-3623~~
~~(underlined), and the end of the measles HA ORF (bolded). The resultant PCR fragment was~~
~~then digested with *A**ff**II* and cloned into p(*A**ff**II* N-P) and p(*A**ff**II* P-M) to create pUC119(HA~~
~~N-P) and pUC119(HA P-M) respectively. pUC119(HA N-P) and pUC119(HA P-M) were~~
~~sequenced over the entire *A**ff**II* insert using the dRhodamine Terminator Cycle Sequencing~~
~~Ready Reaction (ABI prism, PE Applied Biosystems, Foster city, CA), and the sequence was~~
~~confirmed to be correct.~~

The *P**ml* to *B**am**HI* fragments of pUC119(HA N-P) and pUC119(HA P-M) were separately cloned into the full-length antigenome cDNA plasmid p3/7(131)2G+ as

previously described (Durbin et al., *Virology* 235:323-332, 1997a, incorporated herein by reference) to create pFLC(HA N-P) and pFLC(HA P-M) (Figure 1). The *XhoI*-*Ngo*MI fragment (nt 7437-15929) of pFLC*cp45L* was then cloned into the *XhoI*-*Ngo*MI window of both pFLC(HA N-P) and pFLC(HA P-M) to create pFLC*cp45L*(HA N-P) and pFLC*cp45L*(HA P-M). pFLC*cp45L* encodes the three amino acid changes in the L gene of PIV3 *cp45* (aa position 942, 992, and 1558) which confer most of the temperature-sensitivity and attenuation of the *cp45* vaccine candidate virus (Skiadopoulos et al., *J. Virol.* 72:1762-8, 1998, incorporated herein by reference), and the transfer of the *XhoI*-*Ngo*MI fragment transferred those mutations.

10 **Construction of full-length HPIV3 chimeric cDNAs encoding the HA protein of measles in the HN/L junction**

Sub. a b

~~A HPIV3 chimeric cDNA was constructed by PCR to include a heterologous polynucleotide sequence, exemplified by the measles virus HA gene, encoding a heterologous antigenic determinant of the measles virus, flanked by the transcription signals and the noncoding regions of the HPIV3 HN gene. This cDNA was designed to be combined with an rPIV3 vector as an extra gene following the HN gene. First, using Kunkel mutagenesis (Kunkel et al., *Methods Enzymol.* 154:367-382, 1987, incorporated herein by reference), a *StuI* site was introduced in the 3'-noncoding region of the HN gene by mutating the AGACAA sequence at nts 8598-8603 of the antigenome to AGGCCT yielding plasmid p3/7(131)2G-*StuI* (Figure 1B). A cDNA containing the measles HA ORF flanked by HPIV3 sequences (see Figure 1B) was then constructed in three pieces by PCR. The first PCR synthesized the left-hand, upstream piece of the gene. The forward primer 5'-~~

~~GACAATAGGCCTAAAAGGGAAATATAAAAACTTAGGAGTAAAGTTACGCAATCC~~

~~-3' contains a *StuI* site (italicized) followed by HPIV3 sequence (underlined) which includes the downstream end of the HN gene (HPIV3 nts 8602-8620), an intergenic region, and the gene-start signal and sequence from the upstream end of the HN gene (HPIV3 nt 6733-6753).~~

~~The reverse primer 5'-~~

~~GTAGA**ACGCGTT**TATCCGGTCTCGTTGTGGTGACATCTCGAATTGGATTGTCT~~
~~ATTGGGTCCTTCC-3' contains an *MluI* site (italicized) downstream of the start of the~~

~~measles HA ORF (bolded) followed by the complement to HPIV3 nts 6744-6805 (underlined), which are part of the upstream HN noncoding region. The *MluI* site present in the introduced measles virus ORF was created by changing nt 27 from T (in the wild type Edmonston HA gene) to C and nt 30 from C to G. Both of these changes are noncoding in the measles virus~~

ORF. The PCR was performed using p3/7(131)2G-Stu as template. The resulting product, termed PCR fragment 1, is flanked by a *StuI* site at the 5'-end and an *MluI* site at the 3'-end and contains the first 36 nt of the measles HA ORF downstream of noncoding sequence from the HPIV3 HN gene. The second PCR reaction synthesized the right-hand end of the HN gene.

5 The forward primer 5'-

CAGTCACCCGGGAAGATGGAACCAATCGCAGATAGTCATAATTAACCATAATAT
GCATCAATCTATCTATAATAACAA-3' contains the *XmaI* (italics) and the end of the measles HA ORF (bold), followed by HPIV3 nts 8525-8566 (underlined) representing part of the downstream nontranslated region of the HN gene. The reverse primer 5'-

10 CCATGTAATTGAATCCCCCAACACTAGC-3', spans HPIV3 nts 11448-11475, located in the L gene. The template for the PCR was p3/7(131)2G-Stu. PCR fragment 2 which resulted from this reaction contains the last 35 nt of the measles HA ORF and approximately 2800 nt of the L ORF of PIV3 and is flanked by an *XmaI* site and an *SphI* site (which occurs naturally at HPIV3 position 11317). The third PCR reaction amplified the largest, central portion of the
15 measles HA ORF from the template cDNA pTM-7, a plasmid which contains the HA ORF of the Edmonston strain of measles virus supplied by the ATCC. Sequence analysis of this plasmid showed that the measles virus HA ORF contained in PTM-7 contains 2 amino acid differences from pTM-7 ob the Edmonston wild type HA sequence used for insertion into the N-P and M-P junction, and these were at amino acid positions 46 (F to S) and at position 481
20 (Y to N). The forward primer 5'-CGGATAA*ACGCGTTCTACAAAGATAACC-3'* (*MluI* site italicized) and reverse primer 5'-CCATCTT*CCCGGGTGACTGTGCAGC-3'* (*XmaI* site italicized) amplified PCR fragment 3 which contained nts 19-1838 of the measles HA ORF.

To assemble the pieces, PCR fragment 1 was digested with *StuI* and *MluI* while PCR fragment 3 was digested with *MluI* and *XmaI*. These two digested fragments were then cloned by triple
25 ligation into the *StuI-XmaI* window of pUC118 which had been modified to include a *StuI* site in its multiple cloning region. The resultant plasmid, pUC118(HA 1+3) was digested with *StuI* and *XmaI* while PCR fragment 2 was digested with *XmaI* and *SphI*. The two digested products were then cloned into the *StuI-SphI* window of p3/7(131)2G-Stu, resulting in the plasmid pFLC(HA HN-L). The *StuI-SphI* fragment, including the entire measles HA ORF, was then
30 sequenced using the dRhodamine Terminator Cycle Sequencing Ready Reaction (ABI prism, PE Applied Biosystems, Foster city, CA). The chimeric construct sequence was confirmed. In this way, the measles virus HA ORF flanked by HPIV3 transcription signals was inserted as an extra gene into the N/P, P/M, or HN/L junction of an antigenomic cDNA vector comprising a

Sub. 2.6
wild type HPIV3 or into the N/P or P/M junction of an antigenomic cDNA vector comprising an attenuated HPIV3.

Recovery of chimeric rPIV3 wild type and rcp45L expressing the HA protein of measles virus

5 The five full-length vector cDNAs bearing the measles HA ORF as a separate gene were transfected separately into HEp-2 cells on six-well plates (Costar, Cambridge, MA) together with the support plasmids {pTM(N), pTM(P no C), and pTM(L)}, and LipofectACE (Life Technologies), and the cells were simultaneously infected with MVA-T7, a replication-defective vaccinia virus recombinant encoding the bacteriophage T7 polymerase protein as
10 previously described (Durbin et al., Virology 235:323-332, 1997; Durbin et al., Virology 234:74-83, 1997, each incorporated herein by reference). pTM(P no C) is a derivative of pTM(P) (Durbin et al., Virology 261:319-330, 1999) in which the C ORF expression has been silenced by mutation of the C start codon. After incubation at 32°C for three days, the transfection harvest was passaged onto a fresh monolayer of Vero cells in a T25 flask and
15 incubated for 5 days at 32°C (referred to as passage 1). The presence of HPIV3 in the passage 1 harvest was determined by plaque titration on LLC-MK2 monolayer cultures with plaques visualized by immunoperoxidase staining with HPIV3 HN-specific and measles HA-specific monoclonal antibodies as previously described (Durbin et al., Virology 235:323-332, 1997, incorporated herein by reference).

20 The rPIV3(HA HN-L) virus present in the supernatant of the appropriate passage 1 harvest was biologically-cloned by plaque purification three times on LLC-MK2 cells as previously described (Hall et al., Virus Res. 22:173-184, 1992, incorporated herein by reference). rPIV3(HA N-P), rcp45L(HA N-P), rPIV3(HA P-M), and rcp45L(HA P-M) were biologically-cloned from their respective passage 1 harvests by terminal dilution using serial 2-
25 fold dilutions on 96-well plates (12 wells per dilution) of Vero cell monolayers. The biologically-cloned recombinant viruses from the third round of plaque purification or from the second or third round of terminal dilution were then amplified twice in LLC-MK2 cells {rPIV3(HA HN-L) or Vero cells {rPIV3(HA N-P), rcp45L(HA N-P), rPIV3(HA P-M), rcp45L(HA P-M)} at 32°C to produce virus for further characterization. As a first step in
30 confirming and characterizing the recombinant chimeric PIV3s expressing the HA glycoprotein of measles virus, each passage 1 harvest was analyzed by RT-PCR using three different primer pairs; one pair for each location of the HA ORF insert. The first primer pair

amplified a fragment of PIV3 spanning nucleotides 1596-1968 of the full-length HPIV3 genome, which includes the N/P insertion site. This fragment size increased to 2298 nucleotides with the measles HA ORF inserted between the N and P genes. The second primer pair amplified a fragment of PIV3 spanning nucleotides 3438-3866 of the full-length HPIV3 genome, which includes the P/M insertion site. With the measles HA ORF inserted between the P and M genes, this fragment size increased to 2352 nucleotides. The third primer pair amplified a fragment of PIV3 spanning nucleotides 8466-8649 of the full-length antigenome. With the measles HA ORF inserted between the HN and L genes, this fragment size increased to 2211 nucleotides, which includes the HN/L insertion site. All five recovered viruses contained an insert of the appropriate size at the appropriate location. The generation of each PCR product was dependent upon the inclusion of reverse transcriptase, indicating that each was derived from RNA and not from contaminating cDNA.

Monolayers of LLC-MK2 cells in T25 flasks were infected at a multiplicity of infection (MOI) of 5 with either *rcp45L*(HA N-P), *rcp45L*(HA P-M), rJS or were mock infected. Monolayers of Vero cells in T25 flasks were infected with the Edmonston wild type strain of measles virus at an MOI of 5. Vero cell monolayers were chosen for the measles Edmonston virus infection because measles virus does not grow well in LLC-MK2 cells. At 24 hours post-infection, the monolayer was washed with methionine-minus DMEM (Life Technologies). ³⁵S methionine was added to DMEM-minus media at a concentration of 10uCi/ml and 1 ml was added to each flask which was then incubated at 32°C for 6 hours. The cells were harvested and washed 3 times in PBS. The cell pellets were resuspended in 1 ml RIPA buffer {1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100 (Sigma), 0.2% (w/v) SDS, 150mM NaCl, 50mM Tris-HCl, pH 7.4}, freeze-thawed and clarified by centrifugation at 6500 X G for 5 minutes. The cell extract was transferred to a fresh eppendorf tube and a mixture of monoclonal antibodies which recognizes the HA glycoprotein of measles virus (79-XV-V17, 80-III-B2, 81-1-366) (Hummel et al., *J. Virol.* 69:1913-6, 1995; Sheshberadaran et al., *Arch. Virol.* 83:251-68, 1985, each incorporated herein by reference) or which recognizes the HN protein (101/1, 403/7, 166/11) of PIV3 (van Wyke Coelingh et al., *Virology* 160:465-72, 1987, incorporated herein by reference) was added to each sample and incubated with constant mixing for 2 hours at 4°C. Immune complexes were precipitated by adding 200 µl of a 10% suspension of protein A Sepharose beads (Sigma, St. Louis, MO) to each sample followed by constant mixing at 4°C overnight. Each sample was suspended in 90 µl of 1X loading buffer and 10 µl of reducing agent was added. After heating at 70°C for 10 minutes,

20 µl of each sample was loaded onto a 4-12% polyacrylamide gel (NuPAGE, Novex, San Diego, CA) per the manufacturer's recommendations. The gel was dried and autoradiographed (Figure 2). *rcp45L*(HA P-M) and *rcp45L*(HA N-P) encoded a protein precipitated by the anti-measles HA monoclonal antibodies which was the same size as the authentic measles HA protein. *rcp45L*(HA P-M) and *rcp45L*(HA N-P) expressed the measles virus HA protein to a greater extent than did the Edmonston wild type strain of measles virus indicating that these constructs efficiently expressed the measles virus HA from the N/P and P/M junctions of the attenuated strain *rcp45L*. *rcp45L*(HA N-P) and *rcp45L*(HA P-M) were confirmed to be HPIV3-based by their reactivity with the PIV3 anti-HN monoclonal antibodies.

10 **The temperature sensitivity of replication of rPIV3 parent and rPIV3(HA) chimeric viruses *in vitro***

The level of temperature sensitivity of replication of the chimeric rPIV3s bearing the measles virus HA insertion was evaluated to assess whether acquisition of the HA insert modified the level of replication in the chimeric virus compared to the parental, vector virus at various temperatures (Table 1). Serial 10-fold dilutions of *rcp45L*, *rcp45L*(N-P), *rcp45L*(HA P-M), rPIV3(HA HN-L), rPIV3(HA P-M), or rJS were carried out in L-15 supplemented with 5% FBS, 4 mM glutamine, and 50 µg/ml gentamicin on LLC-MK2 cell monolayers in 96 well plates and incubated at 32, 36, 37, 38, 39, or 40°C for 6 days. Virus was detected by hemadsorption and reported as log₁₀TCID₅₀/ml. Interestingly, chimeric derivatives of both wild type vector viruses bearing the measles virus HA gene, rPIV3(HA HN-L) and rPIV3(HA P-M), were slightly restricted in replication at 40°C (Table 1). The two attenuated rPIV3s bearing the measles virus HA gene, *rcp45L*(N-P) and *rcp45L*(HA P-M), possessed a level of temperature sensitivity similar to that of the *rcp45L* parental, vector virus with *rcp45L*(HA P-M) being slightly more ts than its parent. Thus, the viruses bearing the inserts replicated in tissue culture similarly to the parental vector rPIV3 from which they were derived, with only a slight increase in temperature sensitivity. These results indicate that rPIV3 can readily serve as a vector to accommodate the HA insert at different sites without major alteration in replication *in vitro*, and that rPIV3(HA) chimeric viruses can readily accommodate the further addition of one or more attenuating mutations.

Table 1. Replication at permissive and elevated temperatures of recombinant HPIV3s expressing the HA protein of measles virus as an extra gene in the N-P, P-M, or HN-L junctions.

Virus	Virus titer (log ₁₀ TCID ₅₀ /ml) at indicated temperature					
	32°C ¹	36°C	37°C	38°C	39°C	40°C
<i>rcp45L</i> ²	8.2	8.2	7.2	<u>5.2</u> ⁶	3.4	3.0
<i>rcp45L</i> (HA P-M) ³	7.4	6.7	<u>5.2</u>	4.2	1.4	1.4
<i>rcp45L</i> (HA N-P) ³	7.4	7.2	5.7	<u>4.2</u>	2.2	≤1.2
rPIV3(HA HN-L) ⁴	7.7	8.2	7.0	7.7	6.7	<u>5.2</u>
rPIV3(HA P-M) ⁴	7.7	7.4	<u>6.7</u>	6.2	6.2	<u>4.7</u>
PIV3-rJS ⁵	8.7	9.0	9.0	8.4	8.2	9.0

1. Permissive temperature.
2. Recombinant *ts* derivative of the JS wild type strain of HPIV3, bearing 3 attenuating amino acid substitutions derived from *cp45*.
3. Recombinant attenuated *ts* derivative of JS wild type HPIV3 expressing the HA protein of measles virus.
4. Recombinant wild type HPIV3 expressing the HA protein of measles virus.
5. Recombinant wild type HPIV3, strain JS.
6. Underlined titer represents the lowest restrictive temperature at which a 100-fold or greater reduction in titer from that at 32°C is seen and defines the shut-off temperature of the virus.

EXAMPLE II

Chimeric rPIV3s Bearing an Antigenic Determinant of Measles Virus

Replicate Efficiently in Hamsters and Induce High Titers of Antibodies Against Both HPIV3 and Measles

Determination of the level of replication and immunogenicity of the rPIV3(HA) viruses in hamsters

The levels of replication of chimeric rPIV3s bearing an antigenic determinant of the measles virus was compared with that of their parent rPIV3s to determine if the acquisition of the determinant, exemplified by an HA insert, significantly modified their ability to replicate and to induce an immune response *in vivo*. In two different experiments, groups of 6 or 7 4-6 week-old Golden Syrian hamsters were inoculated intranasally with 0.1 ml of EMEM (Life Technologies) containing $10^{6.0}$ PFU of rJS, *rcp45L*, *rcp45L*(HA P-M), *rcp45L*(HA N-P), rPIV3(HA HN-L), or rPIV3(HA P-M) (Tables 2 and 3). On day 4 post-inoculation the hamsters were sacrificed and the lungs and nasal turbinates were harvested. The nasal turbinates and lungs were homogenized in 10% or 20% w/v suspension of L-15 (Quality Biologicals, Gaithersburg, MD) respectively, and the samples were rapidly frozen. Virus present in the samples was titered on 96 well plates of LLC-MK2 cell monolayers and incubated at 32°C for 7 days. Virus was detected by hemadsorption, and the mean \log_{10} TCID₅₀/g was calculated for each group of hamsters. Insertion of the HA gene into wild type rJS (Table 2) restricted its replication 4 to 20-fold in the upper respiratory tract and up to five-fold in the lower respiratory tract indicating only a slight effect of the acquisition of the HA gene on replication of wild type rJS virus in hamsters. The replication of each of the two *rcp45*(HA) antigenic chimeras was 10-fold less in the upper respiratory tract of hamsters (Table 3)-than that of *rcp45L*, the recombinant parent virus bearing the three attenuating mutations in the L protein, but was the same as the *rcp45L* parent in the lower respiratory tract. Thus, for each of the two *rcp45*(HA) antigenic chimeras there was a slight, but statistically significant, reduction in replication in the upper respiratory tract of hamsters indicating that the acquisition of the HA gene by *rcp45L* increased its attenuation for the upper, but not the lower, respiratory tract. Thus, the effect of the insertion of the HA gene on the replication of wild type or attenuated PIV3 was comparable in the upper respiratory tract.

Table 2: Replication of wildtype rPIV3(HA) chimeric viruses in the upper and lower respiratory tract of hamsters

Virus ¹	# Animals	Virus Titer ($\log_{10}\text{TCID}_{50}/\text{gm} \pm \text{S.E.}^2$) [Tukey-Kramer Grouping] ³	
		Nasal Turbinates	Lungs
rcp45L	8	4.0 \pm 0.1[A]	1.5 \pm 0.1[A]
rPIV3(HA N-P)	8	5.1 \pm 0.1[B]	5.9 \pm 0.1[B]
rPIV3(HA P-M)	8	5.9 \pm 0.1[C]	6.7 \pm 0.2[C]
rPIV3(HA HN-L)	8	5.9 \pm 0.2[C]	5.8 \pm 0.1[B]
rJS	8	6.5 \pm 0.1[D]	6.6 \pm 0.2[C]

1. Animals received 10^6TCID_{50} of the indicated virus given intranasally in a 0.1 ml inoculum and the lungs and nasal turbinates were harvested 4 days later.
2. Standard Error.
3. Mean virus titers were assigned to statistically similar groups (A-D) by the Tukey-Kramer test. Therefore, means in each column with different letters are significantly different ($\alpha=0.05$) and those with the same letter are not significantly different.

Table 3: Replication of the rPIV3cp45L(HA) antigenic chimeric viruses in the upper and lower respiratory tract of hamsters

Virus ¹	#Animals	Virus Titer (log ₁₀ TCID ₅₀ /gm±S.E. ²) [Tukey-Kramer Grouping] ³	
		Nasal Turbinates	Lungs
rcp45L	6	4.7±0.2[A]	2.9±0.1[A]
rcp45L(HA N-P)	6	3.7±0.2[B]	2.9±0.1[A]
rcp45L (HA P-M)	7	3.7±0.1[B]	2.9±0.2[A]
rJS	7	6.5±0.1[C]	5.6±0.2[B]

1. Animals received 10⁶ pfu of the indicated virus given intranasally in a 0.1 ml inoculum and the lungs and nasal turbinates were harvested 4 days later.
2. Standard Error.
3. Mean virus titers were assigned to statistically similar groups (A-D) by the Tukey-Kramer test. Therefore, means in each column with different letters are significantly different ($\alpha=0.05$) and those with the same letter are not significantly different.

The ability of the chimeric rHPIV3(HA) viruses to induce an immune response to HPIV3 and to measles virus was studied next. Groups of 6-24 Golden Syrian hamsters (age 4-6 weeks) were infected as described above with either $10^{6.0}$ PFU rJS, rPIV3(HA P-M), *rcp45L*, *rcp45L*(HA P-M), or *rcp45L*(HA N-P) (Table 4) on day 0. Serum was collected from each hamster on day -1 and on day 25 post-inoculation. The serum antibody response to HPIV3 was evaluated by hemagglutination-inhibition (HAI) assay as previously described (van Wyke Coelingh et al., Virology 143:569-582, 1985, incorporated herein by reference), and the serum antibody response to measles virus was evaluated by 60% plaque-reduction assay as previously described (Coates et al., Am. J. Epidemiol. 83:299-313, 1966, incorporated herein by reference). These results were compared with that from an additional control group of cotton rats that received $10^{5.0}$ of the live-attenuated measles virus (Moraten strain) administered intramuscularly on day 0. Cotton rats, rather than hamsters, were used in this group because measles virus is only weakly infectious for hamsters. As can be seen in Table 4, each of the PIV3(HA) chimeric viruses was able to elicit a robust serum neutralizing antibody response against measles virus. There was no significant difference between the amount of serum neutralizing antibody elicited by the attenuated derivative *rcp45L*(HA P-M) as compared to its counterpart in the wild type background, rPIV3(HA P-M). Furthermore, the level of measles virus-neutralizing serum antibodies induced by the rPIV3(HA) recombinants were on average 5-fold greater than that achieved by the intramuscular immunization with the live attenuated measles virus vaccine. In addition, the serum antibody response to HPIV3 produced by all the chimeric viruses was also robust and comparable to that produced by infection with wild type rJS.

Table 4. rPIV3(HA) antigenic chimeric viruses elicit an excellent serum antibody response to both measles virus and PIV3

Virus ¹	# Animals	Serum antibody titer to measles virus (60% plaque reduction neutralization titer, mean reciprocal log ₂ ±S.E. ²)		Sreum antibody response to HPIV3 (HAI titer; mean reciprocal log ₂ ±S.E.)	
		Day 0	Day 25	Day 0	Day 25
<i>rcp45L</i> ³	18	≤3.3±0	≤3.3±0	≤2.0±0	10.7±0.2
<i>rcp45L</i> (HA P-M) ⁴	24	≤3.3±0	12.8±0.1	≤2.0±0	9.2±0.2
<i>rcp45L</i> (HA N-P) ⁵	6	≤3.3±0	13.4±0.4	≤2.0±0	10.8±0.3
rPIV3(HA P-M) ⁶	6	≤3.3±0	13.3±0.3	≤2.0±0	10.3±0.2
Measles virus (Moraten) ⁷	4	≤3.3±0	10.8±0.2	≤2.0±0	≤2.0±0
rJS ⁸	6	≤3.3±0	≤3.3±0	≤2.0±0	10.7±0.2

1. Virus was administered at a dose of 10^{6.0} PFU in a 0.1 ml inoculum intranasally on day 0 to all animals with the exception of those in the measles virus group which received virus by intramuscular injection.
2. Standard Error.
3. Recombinant attenuated HPIV3 with three temperature sensitive (*ts*) mutations in the L protein, derived from *cp45*.
4. Recombinant attenuated HPIV3 in the *cp45L* background with the HA ORF of measles virus in the P/M noncoding region of rPIV3.
5. Recombinant attenuated HPIV3 in the *cp45L* background with the HA ORF of measles virus in the N/P noncoding region of rPIV3.
6. Recombinant HPIV3 with the HA ORF of measles virus in the P/M noncoding region of wild type rPIV3.
7. The live attenuated measles vaccine virus, Moraten strain, was administered at a dose of 10⁵ pfu in a 0.1 inoculum by IM injection to 4 cotton rats in a separate study. All other animals were hamsters.
8. Recombinant wildtype HPIV3.

Six hamsters from each group and from a control group similarly infected with RSV were challenged on day 25 with $10^{6.0}$ pfu of biologically-derived HPIV3 wildtype virus given intranasally in a 0.1 ml inoculum. The lungs and nasal turbinates were harvested on day 4 and processed as described above. Virus present in the samples was titrated on 96 well plates of LLC-MK2 cell monolayers and incubated at 32°C for 7 days. Virus was detected by hemadsorption and the mean \log_{10} TCID₅₀/g was calculated for each group of hamsters. As shown in Table 5, those hamsters which had received the chimeric viruses, whether in the attenuated or wild type backbone, were highly protected against replication of challenge wild type HPIV3 in both the upper and the lower respiratory tract. Thus, despite the slight attenuating effect of the acquisition of the measles virus HA gene on replication of the *rcp45*(HA) chimeric viruses, infection with either *rcp45*L(HA P-M) or *rcp45*L(HA N-P) induced a high level of protection against HPIV3 as indicated by approximately a 1000-fold reduction of its replication in the upper and lower respiratory tract of hamsters. Since wild type measles virus does not replicate efficiently in hamsters, it cannot be used to challenge this host. However, it is expected that the attenuated chimeric *rcp45*L(HA) vaccine candidates will be highly efficacious against measles virus since high levels of neutralizing antibody, ie., mean titer of greater than 1:5000, were induced. Comparable levels of measles virus antibodies are associated with strong resistance to measles virus disease in humans (Chen et al., J. Infect. Dis. 162:1036-42, 1990, incorporated herein by reference).

Table 5. Attenuated and wildtype HPIV3-measles HA chimeric viruses are highly protective against replication of challenge wildtype PIV3 in the upper and lower respiratory tracts of hamsters.

Animals Immunized with ¹	# Animals	Virus titer (log ₁₀ TCID ₅₀ /g) [Tukey-Kramer Grouping ³] Reduction in Titer (log ₁₀)			
		Nasal Turbinates	Lungs	Nasal Turbinates	Lungs
RSV	6	7.0±0.3[A]	5.7±0.4[A]	NA ²	NA
rcp45L(HA P-M)	6	3.4±0.3[B]	2.9±0.0[B]	3.6	2.8
rcp45L(HA N-P)	6	2.6±0.3[B]	3.4±0.2[B]	4.4	2.3
rPIV3(HA P-M)	6	2.0±0.3[B]	3.2±0.1[B]	5.0	2.5
rcp45L	6	1.9±0.2[B,C]	3.6±0.1[B]	5.1	2.1
rJS	6	<1.4±0.0[C]	2.9±0.2[B]	>5.7	2.8

1. All groups were challenged with 10⁶ pfu biologically-derived JS wildtype PIV3 in a 0.1 ml inoculum given intranasally.
2. Not applicable.
3. Mean virus titers were assigned to statistically similar groups (A-C) by the Tukey-Kramer test. Therefore, means in each column with different letters are significantly different ($\alpha=0.05$) and means with the same letter are not significantly different.

EXAMPLE III

Construction of Antigenomic cDNAs Encoding a Chimeric HPIV3-1 Vector Bearing a HPIV2 HN Gene as an Extra Transcription/Translation Unit Inserted Between the F and HN Genes, and Recovery of Infectious Viruses

5 ~~rPIV3-1 is a recombinant chimeric HPIV3 in which the HN and F genes have~~
been replaced by those of HPIV1 (see, e.g., Skiadopoulos et al., Vaccine 18:503-510, 1999,
Tao et al., Vaccine 17:1100-1108, 1999; U.S. Patent Application Serial No. 09/083,793, filed
May 22, 1998, each incorporated herein by reference). In the present example, the HN gene of
HPIV2 was inserted into the rPIV3-1 chimeric virus that served as a vector to produce a
10 chimeric derivative virus, bearing an introduced heterologous antigenic determinant from
HPIV2, able to protect against both HPIV1 and HPIV2. The HPIV2 HN gene also was
inserted into an attenuated derivative of rPIV3-1, designated rPIV3-1~~cp45~~, which contains 12
of the 15 ~~cp45~~ mutations, i.e., those mutations on genes other than HN and F, inserted into the
rPIV3 backbone (Skiadopoulos et al., Vaccine 18:503-510, 1999). The source of the HPIV2
15 wild type virus was the wild type strain V9412-6 (designated PIV2/V94) (Tao et al., Vaccine
17:1100-1108, 1999), which was isolated in Vero cells from a nasal wash that was obtained in
1994 from a child with a natural HPIV2 infection. PIV2/V94 was plaque purified 3 times on
Vero cells before being amplified twice on Vero cells using OptiMEM tissue culture medium
without FBS. A cDNA clone of the HN gene of PIV2/V94 was generated from virion RNA by
20 reverse transcription (RT) using random hexamers and Superscript Preamplification System
(Life Technologies) followed by PCR using Advantage cDNA Synthesis kit (Clontech, Palo
Alto, CA) and synthetic primers which introduced *NcoI*-*HindIII* sites flanking the HN cDNA
(Figure 3A). The sequences of these primers were: (with HPIV specific sequences in upper
case, restriction sites underlined, nts which are non-HPIV or which are altered from wt in
25 lower case, and start and stop codons in bold), upstream HPIV2 HN 5'-
gggccATGGAAGATTACAGCAAT-3'; downstream HPIV2 HN 5'-
caataagcTTAAAGCATTAGTTCCC-3'. The HN PCR fragment was digested with *NcoI*-
HindIII and cloned into pLit.PIV31HNhc to generate pLit.32HNhc (Figure 3 B). The HPIV2
HN heterologous gene insert in pLit.32HNhc was completely sequenced using the
30 ThermoSequenase Kit and ³³P-labeled terminators (Pharmacia Amersham, Piscataway, NJ) and
was confirmed to contain the authentic sequence of the PIV2/94 HN coding region.

The HPIV2 HN gene in pLit.32HNhc was further modified by PCR and Deep Vent thermostable DNA polymerase (New England Biolab, Beverly, MA) to introduce *Ppu*MI sites for cloning into the unique *Ppu*MI site in p38'ΔPIV31hc, Figure 3C (Skiadopoulos et al., Vaccine 18:503-510, 1999). The sequences of these primers were (with HPIV specific

5 sequences in upper case, relevant restriction sites underlined, non-HPIV nt or nt altered from wt in lower case): upstream HPIV2 HN

5'-gcgatgggcccGAGGAAGGACCCAATAGACA-3'; downstream HPIV2 HN

5'-cccgggtcctgATTTC~~CCG~~GAGCAGCTTTG-3'. The modified cDNA bearing the HPIV2 HN ORF consists of (from left to right) a partial 5'-untranslated region (5'-UTR) of HPIV3 HN including the *Ppu*MI site at the 5'-end, the HPIV2 HN ORF, the 3'-UTR of HPIV3 HN, a complete set of HPIV3 transcription signals (i.e. gene stop, intergenic region and gene start sequences) whose sequences match those at the HPIV3 HN and L gene junction, a partial 5'-UTR of HPIV3 L, and an added *Ppu*MI site at its 3'-end (Figure 3C). This fragment was digested with *Ppu*MI and inserted into p38'ΔPIV31hc digested with *Ppu*MI to generate 15 p38'ΔPIV31hc.2HN (Figure 3D). The inserted *Ppu*MI cassette was sequenced in full and found to be as designed. The insert from p38'ΔPIV31hc.2HN was isolated as a 8.5 kb BspEI-SphI fragment and introduced into the BspEI-SphI window of pFLC.2G+.hc or pFLC*cp*45 to generate pFLC.31hc.2HN or pFLC.31hc.*cp*45.2HN, respectively (Figure 3, E and F). pFLC.2G+.hc and pFLC*cp*45 are full-length antigenomic clones encoding wt rPIV3-1 and 20 rPIV3*cp*45, respectively, as described previously (Skiadopoulos et al., J. Virol. 73:1374-81, 1999; Tao et al., J. Virol. 72:2955-2961, 1998, each incorporated herein by reference).

Confluent HEP-2 cells were transfected with pFLC.31hc.2HN or pFLC.3-1hc.*cp*45.2HN plus the pTM(N), pTM(P no C), and pTM(L) support plasmids in the presence of MVA-T7 as previously described (Durbin et al., Virology 235:323-332, 1997, incorporated 25 herein by reference). The recombinant chimeric viruses recovered from transfection were activated by addition of TPCK trypsin (Catalog No. 3741, Worthington Biochemical Corp., Freehold, NJ) as were all passages and titrations of viruses bearing the HPIV1 HN and F glycoproteins as described previously (Tao et al., J. Virol. 72:2955-2961, 1998, incorporated herein by reference). Recovered chimeric recombinant viruses rPIV3-1.2HN and rPIV3-1*cp*45.2HN were purified by plaque-to-plaque-to-plaque passage on LLC-MK2 monolayer in 30 agarose overlay as previously described (Tao et al., Vaccine 17:1100-1108, 1999, incorporated herein by reference).

To determine if the rPIV3-1.2HN and rPIV3-1cp45.2HN recombinants contain the heterologous HPIV2 HN gene, viral RNA from each recovered recombinant chimeric virus was amplified on LLC-MK2 cells and concentrated by polyethylene glycol (PEG) precipitation (Mbiguino et al., J. Virol. Methods 31:161-170, 1991, incorporated herein by reference).

5 Virion RNA (vRNA) was extracted with Trizol (Life Technologies) and used as template to synthesize first strand cDNA using Superscript Preamplification system (Life Technologies, Gaithersburg, MD) and random hexamer primers as described above. The synthesized cDNA was amplified by PCR with the Advantage cDNA Synthesis kit (Clontech, Palo Alto, CA) with primers specific for HPIV1 F and HPIV1 HN coding region (for HPIV1 F 5'-

10 AGTGGCTAATTGCATTGCATCCACAT-3' and for HPIV1 HN 5'-GCCGTCTGCATGGTGAATAGCAAT-3'). The relative locations of the PIV1 F and HN primers are indicated by arrows in Figures 3 and 4. Amplified DNA fragments were digested and analyzed on agarose gels (Figure 4). Data for rPIV3-1cp45.2HN is not shown, but was comparable and confirmed in structure. rPIV3-1.2HN and rPIV3-1cp45.2HN each contained
15 the insert of the expected size, and the digestion patterns with a number of restriction enzymes confirmed the identity and authenticity of the inserts. The presence of the cp45 mutations in rPIV3-1cp45.2HN was also confirmed.

To confirm the expression of HPIV2 HN by the rPIV3-1.2HN chimeric virus, LLC-MK2 monolayers in T25 flasks were infected with PIV2/V94, rPIV3-1, or rPIV3-1.2HN
20 at a MOI of 5 in 5 ml of serum-free OptiMEM containing 0.5 µg/ml TPCK trypsin. After incubation for 18 hours at 32°C, the flasks were washed three times with 5 ml of methionine and cysteine deficient DMEM (BioWhittaker, Walkersville, MD). Cells were then fed with 1 ml of methionine and cysteine deficient DMEM supplemented with 120 µCi of ProMix 35S-methionine and 35S-cysteine mixture (Pharmacia Amersham, Piscataway, NJ) and incubated
25 for 18 hours at 32°C. Cells were scraped into medium, pelleted by brief centrifugation in a microfuge, and washed three times with cold PBS. Each cell pellet was resuspended in 1 ml RIPA buffer (1% sodium deoxycholate, 1% Triton X-100, 0.2% SDS, 150 mM NaCl, and 50 mM Tris-HCl, pH7.4) containing 250 units/ml of Benzonase (Sigma), freeze/thawed once, and clarified by centrifugation at 12,000 X g for 5 min in a microfuge. Clarified supernatants were
30 transferred to a clean microfuge tube, mixed with 50 µl of anti-HPIV2 HN monoclonal antibody (mAb) 150S1 (Tsurudome et al., Virology 171:38-48, 1989, incorporated herein by reference), and incubated with mixing at 4°C for 3 hours. The monoclonal antibody was precipitated by the addition to each tube of 0.2 ml of 10% Protein A sepharose suspension (in

RIPA buffer) and incubation with mixing at 4° for 18 hours. The beads were washed three times with RIPA buffer and pelleted by brief centrifugation in a microfuge. Each sample was suspended in 90 µl of 1X loading buffer, and 10 µl was resolved on a 4-12% SDS polyacrylamide gel (PAGE; NOVEX, San Diego, CA). The gel was dried and
5 autoradiographed (Figure 5). The mAb, specific to PIV2 HN, precipitated a protein from both rPIV3-1.2HN and PIV2/V94 infected LLC-MK2 cells, but not from rPIV3-1-infected cells, with a size expected for the 86kD Kd HN protein of HPIV2 (Rydbeck et al., J. Gen. Virol. 69:931-5, 1988, incorporated herein by reference).

10 EXAMPLE IV

The rPIV3-1 Viruses Carrying an HPIV2 Antigenic Determinant Exhibit Temperature Sensitive Phenotypes Similar to Those of Their Parental Vector Viruses

The level of temperature sensitivity of replication of rPIV3-1.2HN and rPIV3-1.cp45.2HN in LLC-MK2 cells was evaluated to determine if the acquisition of the HN ORF
15 of HPIV2 by rPIV3-1 wild type or attenuated viruses employed as vectors altered the level of temperature sensitivity of replication in the resultant chimeric derivatives bearing the heterologous antigenic determinant of HPIV2 compared to the parental, vector viruses (Table 6). rPIV3-1.2HN and rPIV3-1.cp45.2HN, along with control viruses, were serially diluted 1:10 in 1X L15 supplemented with 0.5 µg/ml TPCK trypsin and used to infect LLC-MK2
20 monolayers in 96 well plates in quadruplicate. Infected plates were placed at various temperatures for 7 days before the virus titers were determined by hemadsorption using 0.2% guinea pig erythrocytes (in 1X PBS). The virus titers are presented as log₁₀TCID₅₀ ± standard error (S.E.). As shown in Table 6, rPIV3-1.2HN and rPIV3-1.cp45.2HN exhibited a level of temperature sensitivity similar to that of their parental, vector viruses, i.e. rPIV3-1 and rPIV3-1.cp45, respectively, each of which lacks the HPIV2 HN insert. This indicated that the
25 introduction of one extra transcription/translation unit in rPIV3-1.2HN and rPIV3-1.cp45.2HN, does not significantly alter their level of temperature sensitivity of replication *in vitro*.

Table 6. The rPIV3-1 viruses carrying the PIV2 HN insertion have a temperature sensitive phenotype similar to that of their parental virus.

Virus	Titer at 32° C ^a (log ₁₀ TCID ₅₀)		Titer reduction (log ₁₀ TCID ₅₀) at various temperatures (°C) ^a					
			35° ^b	36°	37°	38°	39°	40°
PIV2/V9412	7.8	0.3	(0.1) ^c	0.0	(0.4)	(0.4)	0.0	
PIV1/Wash64	8.5	1.5	1.1	1.4	0.6	0.5	0.9	
rPIV3/JS	7.9	0.3	0.1	0.1	(0.3)	(0.4)	0.4	
PIV3 <i>cp45</i>	7.8	0.5	0.3	1.3	<u>3.4</u> ^d	6.8	6.9	
rPIV3-1	8.0	0.8	0.5	0.6	0.9	1.1	<u>2.6</u>	
rPIV3-1.2HN	8.3	0.5	(0.3)	0.3	0.6	1.5	<u>2.6</u>	
rPIV3-1 <i>cp45</i>	8.0	0.5	0.4	<u>3.4</u>	4.8	6.6	7.5	
rPIV3-1 <i>cp45.2HN</i>	8.0	0.3	1.4	<u>2.9</u>	5.3	7.6	7.6	

^a Data presented are means of two experiments.

^b Data at 35°C were from single experiment.

^c Numbers in parentheses represent titer increase.

^d Underlined value indicates shut-off temperature at which the virus titer showed a reduction of 100-fold or more in comparison to the titer at 32°C.

EXAMPLE V

Replication and Immunogenicity of rHPIV3-1.2HN Chimeric Viruses in Animals

To determine the level of replication of the chimeric viruses *in vivo*, Golden Syrian hamsters in groups of six were inoculated intranasally with 0.1 ml of 1X L-15 medium containing $10^{5.3}$ TCID₅₀ (or 10^6 pfu) of virus (Table 7). Four days after infection, hamsters were sacrificed and their lungs and nasal turbinates harvested. Virus titers, expressed as mean \log_{10} TCID₅₀/gram of tissue (Table 7), were determined. rPIV3-1 expressing the PIV2 HN gene, termed rPIV2-1.2HN, is more restricted in replication than its rPIV3-1 parent as indicated by a 30-fold reduction in virus titer in both the upper and lower respiratory tracts of hamsters. Thus, the insertion of a transcription/translation unit expressing the PIV2 HN protein into rPIV3-1 attenuates the virus for hamsters. The attenuating effect of insertion of a transcription/translation unit containing PIV2 HN ORF into rPIV3-1 was slightly more than that observed for the insertion of a similar unit containing the measles HA ORF into the recombinant JS strain of wild type PIV3. The rPIV3-1*cp45*.2HN virus was 1,000-fold more restricted in replication than the rPIV3-1*cp45* parent indicating that the attenuating effect of the PIV2 HN insertion and the *cp45* mutations are additive. It should be possible to adjust the level of attenuation as needed by adding fewer *cp45* mutations than the 12 that are present in rPIV3-1.*cp45*.2HN.

Table 7. The chimeric rPIV3-1 expressing the HN glycoprotein of PIV2 (rPIV3-1.2HN) is attenuated in the respiratory tract of hamsters

Experiment No.	Virus	Virus titer in indicated tissue $\log_{10}\text{TCID}_{50}/\text{g} \pm \text{S.E.})^c$	
		NT	Lungs
1 ^a	rPIV3-1	$6.9 \pm 0.1[\text{A}]^d$	$6.0 \pm 0.3[\text{A}]$
	rPIV3-1.2HN	$5.4 \pm 0.2[\text{B}]$	$4.4 \pm 0.4[\text{C}]$
2 ^b	rPIV3-1	$6.7 \pm 0.1[\text{A}]$	$6.6 \pm 0.2[\text{A}]$
	rPIV3-1.2HN	$5.1 \pm 0.1[\text{B}, \text{C}]$	$5.2 \pm 0.2[\text{B}]$
	rPIV3-1cp45	$4.6 \pm 0.3[\text{C}]$	$1.8 \pm 0.4[\text{D}]$
	rPIV3-1cp45.2HN	$1.5 \pm 0.1[\text{D}]$	$\leq 1.2[\text{D}]$
	rPIV3/JS	$6.5 \pm 0.2[\text{A}]$	$6.7 \pm 0.1[\text{A}]$
	rcp45	$4.9 \pm 0.2[\text{B}, \text{C}]$	$1.2 \pm 0.04[\text{D}]$

^a Groups of six animals were inoculated intranasally with 10^6 pfu of indicated virus in 0.1 ml medium on day 0.

^b Groups of 6 hamsters were inoculated intranasally as in Experiment 1 with $10^{5.3}$ TCID₅₀ of indicated virus on day 0.

^c Lungs and nasal turbinates of the hamsters were harvested on day 4. Virus titers in tissue were determined and the titer expressed as $\log_{10}\text{TCID}_{50}/\text{gram} \pm$ standard error (S.E.). NT = nasal turbinates.

^d Means in each column with a different letter are significantly different ($\alpha=0.05$) by Duncan's Multiple Range test whereas those with the same letter are not significantly different.

Since the single rPIV3-1.2HN virus expresses protective antigens of PIV1 (the F and HN glycoprotein) and PIV2 (the HN glycoprotein only), infection with this virus will induce resistance against challenge with either PIV1 or PIV2 wild type viruses. To verify this, Golden Syrian hamsters in groups of 12 were immunized intranasally with $10^{5.3}$ TCID₅₀ of virus as described above. Half of the hamsters were challenged with PIV2 on day 29, the remaining half with PIV1 on day 32. Hamster lung and nasal turbinate tissues were harvested 4 days after challenge, and titer of challenge virus were determined as described above (Table 8). Sera were obtained before and 28 days after immunization and tested for their neutralizing antibody titer against PIV1 and PIV2.

Table 8. The chimeric rPIV3-1 virus expressing the HN glycoprotein of PIV2 (rPIV3-1.2HN) protects hamsters against challenge with both PIV1 and PIV2

Immunizing virus ^a	Serum neutralizing antibody titer against indicated virus (reciprocal mean log ₂ ±SE) ^b			Titer of challenge virus in indicated tissues (log ₁₀ TCID ₅₀ /g±SE) ^c			
	PIV1		PIV2	PIV1		PIV2	
	pre	post	pre	post	NT	Lung	Lung
rPIV3/JS	≤4.0±0.0	≤4.0±0.0	4.5±0.1	4.6±0.2	5.4±0.2	5.1±0.1	6.8±0.2
PIV2	≤4.0±0.0	≤4.0±0.0	4.3±0.2	9.6±0.2	5.7±0.2	5.7±0.2	≤1.2
rPIV3-1	4.2±0.1	8.5±0.3	4.0±0.0	4.2±0.1	≤1.2	≤1.2	6.5±0.2
rPIV3-1.2HN	≤4.0±0.0	6.2±0.2	4.1±0.1	8.3±0.2	2.3±0.5	≤1.2	≤1.2
rPIV3-1cp45	≤4.0±0.0	6.2±0.4	≤4.0±0.0	4.0±0.0	3.6±0.3	2.7±0.5	5.7±0.4
rPIV3-1cp45.2HN	4.0±0.9	4.1±0.1	4.0±0.0	4.2±0.1	5.1±0.2	4.8±0.2	6.6±0.2

^a Hamsters in groups of 12 were immunized with 10^{5.3} TCID₅₀ of indicated virus intranasally on day 0.

^b Serum was diluted 1:10 with OptiMEM and heat-inactivated by incubation at 56° for 30 min. The serum neutralizing antibody titer was determined on LLC-MK2, and the titers are expressed as reciprocal mean log₂±standard error (SE).

^c Half of the hamsters from each immunized group were challenged with 10⁶ TCID₅₀ PIV2 on day 29, and the remaining half were challenged with 10⁶ TCID₅₀ PIV1 on day 32. Tissue samples were harvested 4 days after challenge, and challenge virus titers are expressed as log₁₀TCID₅₀/gram of tissue ± SE. NT = nasal turbinates.

As expected PIV3 provided no resistance against either PIV1 or PIV2, while previous infection with PIV2 wild type virus and rPIV3-1 induced complete resistance to replication of PIV2 and PIV1 challenge viruses, respectively. In contrast to these viruses that provided protection against only one virus, rPIV3-1.2HN induced antibody to both PIV1 and PIV2 and included strong resistance to both PIV1 and PIV2 as indicated by the 1,000- to 10,000-fold reduction in replication of each virus in the upper and lower respiratory tract of rPIV3-1.2HN immunized hamsters. This indicated that a single recombinant chimeric PIV can induce resistance against two human viral pathogens. However, the derivative of rPIV3-1.2HN carrying the *cp45* mutations failed to induce significant resistance to replication of wild type PIV1 or PIV2 challenge virus indicating that this particular recombinant chimeric virus is over-attenuated in hamsters. Introduction of one or several selected *cp45* mutations, rather than the complete set of 12 mutations, into rPIV3-1.2HN can be done to adjust the level of attenuation of rPIV3-1.2HN if necessary.

EXAMPLE VI

Construction and Characterization of Chimeric HPIV3-2 Vaccine Recombinants

Expressing Chimeric Glycoproteins

The present example details development of a live attenuated PIV2 candidate vaccine virus for use in infants and young children using reverse genetic techniques. Preliminary efforts to recover recombinant chimeric PIV3-PIV2 virus carrying full-length PIV2 glycoproteins in a wild type PIV3 backbone, as described above for HPIV3-1 chimeric constructs, did not yield infectious virus. However, viable PIV2-PIV3 chimeric viruses were recovered when chimeric HN and F ORFs rather than full-length PIV2 ORFs were used to construct the full-length cDNA. The recovered viruses, designated rPIV3-2CT in which the PIV2 ectodomain and transmembrane domain was fused to the PIV3 cytoplasmic domain and rPIV3-2TM in which the PIV2 ectodomain was fused to the PIV3 transmembrane and cytoplasmic tail domain, possessed similar, although not identical, *in vitro* and *in vivo* phenotypes. Thus, it appears that only the cytoplasmic tail of the HN or F glycoprotein of PIV3 is required for successful recovery of PIV2-PIV3 chimeric viruses.

The rPIV3-2 recombinant chimeric viruses exhibit a strong host range phenotype, i.e. they replicate efficiently *in vitro* but are strongly restricted in replication *in vivo*. This attenuation *in vivo* occurs in the absence of any added mutations from *cp45*.

Although rPIV3-2CT and rPIV3-2TM replicated efficiently *in vitro*, they were highly attenuated in both the upper and the lower respiratory tract of hamsters and African green monkeys (AGMs), indicating that chimerization of the HN and F proteins of PIV2 and PIV3 itself specified an attenuation phenotype *in vivo*. A phenotype including efficient replication *in vitro* and highly restricted growth *in vivo* is greatly desired for vaccine candidates. Despite this attenuation, they were highly immunogenic and protective against challenge with PIV2 wild type virus in both species. rPIV3-2CT and rPIV3-2TM were further modified by the introduction of the 12 PIV3 *cp45* mutations located outside of the HN and F coding sequences to derive rPIV3-2CT_{*cp45*} and rPIV3-2TM_{*cp45*}. These derivatives replicated efficiently *in vitro* but were even further attenuated in hamsters and AGMs indicating that the attenuation specified by the glycoprotein chimerization and by the *cp45* mutations was additive. These findings identify the rPIV3-2CT and rPIV3-2TM recombinants as preferred candidates for use in live attenuated PIV2 vaccines.

Viruses and cells

The wild type PIV1 strain used in this study, PIV1/Washington/20993/1964 (PIV1/Wash64) (Murphy et al., Infect. Immun. 12:62-68, 1975, incorporated herein by reference), was propagated in LLC-MK2 cells (ATCC CCL 7.1) as previously described (Tao et al., J. Virol. 72:2955-2961, 1998, incorporated herein by reference). The PIV wild type virus, strain V9412-6, designated PIV2/V94, was isolated in qualified Vero cells from a nasal wash of a sick child in 1994. PIV2/V94 was plaque purified three times on Vero cells before being amplified twice on Vero cells using OptiMEM without FBS. The wild type cDNA-derived recombinant PIV3/JS strain (rPIV3/JS) was propagated as previously described (Durbin et al., Virology 235:323-332, 1997, incorporated herein by reference). The modified vaccinia Ankara virus (MVA) recombinant that expresses the bacteriophage T7 RNA polymerase was generously provided by Drs. L. Wyatt and B. Moss (Wyatt et al., Virology 210:202-205, 1995, incorporated herein by reference).

HEp-2 cells (ATCC CCL 23) were maintained in MEM (Life Technologies, Gaithersburg, MD) with 10% fetal bovine serum, 50 µg/ml gentamicin sulfate, and 2 mM glutamine. Vero cells below passage 150 were maintained in serum-free medium VP-SFM (Formula No. 96-0353SA, Life Technologies) with 50 µg/ml gentamicin sulfate and 2 mM glutamine.

Virion RNA isolation, reverse transcription and PCR amplification of viral genes, and automated sequencing

To clone viral genes or to verify genetic markers of recombinant chimeric viruses, viruses were amplified on cultured cells and concentrated by polyethylene glycol precipitation as previously described (Mbiguino et al., *J. Virol. Methods* 31:161-170, 1991, incorporated herein by reference). Virion RNA was extracted from the virus pellet using Trizol reagent (Life Technologies) and used as template for reverse transcription (RT) with the Superscript Preamplification system (Life Technologies). The cDNA was further PCR amplified using the Advantage cDNA kit (Clontech, Palo Alto, CA). For cloning or sequencing purposes, the RT-PCR amplified DNA was purified from agarose gels using NA45 DEAE membrane as suggested by the manufacturer (Schleicher & Schuell, Keene, NH). Sequencing was performed with the dRhodamine dye terminator cycling sequencing kit (Perkin Elmer, Foster City, CA) and an ABI 310 Gene Analyzer (Perkin Elmer, Foster City, CA).

Construction of the chimeric PIV3-PIV2 antigenomic cDNAs encoding the complete PIV2 F and HN proteins or chimeric F and HN proteins containing a PIV2-derived ectodomain and PIV3-derived cytoplasmic tail domain

Sub. c10
A DNA encoding a full-length PIV3 antigenomic RNA was constructed in which the PIV3 F and HN ORFs were replaced by their PIV2 counterparts following the strategy described previously (Tao et al., *J. Virol.* 72:2955-2961, 1998) for PIV3-PIV1. Details of this construction are presented in Figure 6. PIV2/V94 propagated in Vero cells was concentrated and virion RNA (vRNA) was extracted from the virus pellet using Trizol reagent. The F and HN ORFs of PIV2/V94 were reverse transcribed from vRNA using random hexamer primers and the SuperScript Preamplification System before being amplified by PCR using the cDNA Advantage kit and primer pairs specific to PIV2 F and HN genes, respectively (1, 2 and 3, 4; Table 9). The amplified cDNA fragment of PIV2 F ORF was digested with NcoI plus BamHI and ligated into the NcoI-BamHI window of pLit.PIV31.Fhc (Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference) to generate pLit.PIV32Fhc. The BspEI site in the PIV3 full-length cDNA is unique and we planned to use it to exchange segments between cDNAs (see Figures 6-8). Therefore, a BspEI site that was found in the PIV2 F ORF was removed by site-directed mutagenesis without affecting the amino acid sequence. The cDNA fragment of PIV2 HN ORF was digested with NcoI plus HindIII and ligated into the NcoI-HindIII window of pLit.PIV31.HNhc (Tao et al., *J. Virol.* 72:2955-2961, 1998) to generate pLit.PIV32HNhc. The PIV2 ORFs in pLit.PIV32Fhc and pLit.PIV32HNhc

were sequenced, and the sequence was found to be as designed. The nucleotide sequences for the PIV2 F and HN ORFs are submitted in the GenBank (Accession No. pending).

pLit.PIV32Fhc and pLit.PIV32HNhc were each digested with PpuMI plus SpeI and assembled to generate pLit.PIV32hc. The 4 kb BspEI-SpeI fragment of pLit.PIV32hc was introduced into

5 the BspEI-SpeI window of p38'ΔPIV31hc (Skiadopoulos et al., Vaccine 18:503-510, 1999,

incorporated herein by reference) to generate p38'ΔPIV32hc. The 6.5 kb fragment, generated

by BspEI and SphI digestion of p38'ΔPIV32hc, containing the PIV2 full-length F and HN

ORFs was introduced into the BspEI-SphI window of pFLC.2G+.hc (Tao et al., J. Virol.

72:2955-2961, 1998) to generate pFLC.PIV32hc (Figure 6; Table 10 = SEQ ID NO:).

Sub. a.10

107

Table 9. Primers used in construction of PIV3-2 full-length chimeric antigenomic cDNAs

Primer No.	Gene	Direction	Position		Used in the construction or characterization of:	Sequence ^a
			Beginning	End		
1	PIV2 F	sense	PIV2 F start codon 5070 ^b	20 bp down stream 5091	pFLC.PIV32hc	gtaccATGgATCACCTGCATCCAAT
2	PIV2 F	antisense	PIV2 F stop codon 6732 ^b	20 bp upstream 6705 ^b	pFLC.PIV32hc	tgtggatccTAAGATATATCCCATATATGTTTC
3	PIV2 HN	sense	PIV2 HN start codon 6837 ^b	18 bp down stream 6856 ^b	pFLC.PIV32hc	ggggccATGGAAGATTACAGCAAT
4	PIV2 HN	antisense	PIV2 HN stop codon 8558 ^b	17 bp upstream 8538 ^b	pFLC.PIV32hc	caataagcTTAAAGCATTAGTTCC
5	PIV2 F	sense	5069 ^c	5088 ^c	pFLC.PIV32TM	ATGCATCACCTGCATCCAAT
6	PIV2 F	antisense	6538 ^c	6517 ^c	pFLC.PIV32TM	TAGTGAATAAAAGTGCTTGGCT
7	PIV2 HN	sense	6962 ^c	6985 ^c	pFLC.PIV32TM	CATGAGATAAATTCATCTTGATGTT
8	PIV2 HN	antisense	8560 ^c	8537 ^c	pFLC.PIV32TM	agcTTAAAGCATTAGTTCCTTAA
9	PIV3 F	sense	6539 ^c	6566 ^c	pFLC.PIV32TM	ATCATAAATTAATTTTGATAAATGATCATTA
10	PIV3 F	antisense	5068 ^c	5050 ^c	pFLC.PIV32TM	GTTTCAGTGCCTTGTGTGTT
11	PIV3 HN	sense	8561 ^c	8587 ^c	pFLC.PIV32TM	TCATAATTAACCATATAATATGCATCAAT
12	PIV3 HN	antisense	6961 ^c	6938 ^c	pFLC.PIV32TM	GATGGAATTAATTAGCACTATGAT
13	PIV2 F	sense	5069 ^d	5088 ^d	pFLC.PIV32CT	ATGCATCACCTGCATCCAAT
14	PIV2 F	antisense	6607 ^d	6589 ^d	pFLC.PIV32CT	GATGATGTAGGCAATCAGC

Sub a12

15	PIV2 HN	sense	6887 ^d	6904 ^d	pFLC.PIV32CT	ACTGCCACAATTCTTGGC
16	PIV2 HN	antisense	8536 ^d	8511 ^d	pFLC.PIV32CT	TTAAAGCAATTAGTTCCTTAAATAATG
17	PIV3 F	sense	6620 ^d	6642 ^d	pFLC.PIV32CT	AAGTATTACAGAAATTCAAAAGAG
18	PIV3 F	antisense	5068 ^d	5050 ^d	pFLC.PIV32CT	GTTCAAGTGCTTGTGTGTT
19	PIV3 HN	sense	8525 ^d	8551 ^d	pFLC.PIV32CT	TCATAATTAAACCATAAATATGCATCAAT
20	PIV3 HN	antisense	6898 ^d	6879 ^d	pFLC.PIV32CT	CTTATTAGTGAGCTTGTGTC
21	PIV2 F	Sense	6608 ^{c,d}	6630 ^{c,d}	Chimera confirmation	ACCGCAGCTGTAGCAATAGT
22	PIV2 HN	antisense	7522 ^c 7501 ^d	7502 ^c 7481 ^d	Chimera confirmation	GATTCCATCACTTAGGTAAAT
23	PIV3 M	sense	4759 ^{c,d}	4780 ^{c,d}	Chimera confirmation	GATACTATCCTAATATTATTGC
24	PIV3 L	antisense	9100 ^c 9076 ^d	9081 ^c 9057 ^d	Chimera confirmation	GCTAAATTTTGATAGCACATT

^a All the primers are annotated in that the PIV specific sequences are in uppercase, non-PIV sequences in lowercase, start and stop codons in bold, and restriction sites underlined.
^b The numbers are the nt positions in the full-length antigenomic cDNA construct pFLC.PIV32hc.
^c The numbers are the nt positions in the full-length antigenomic cDNA construct pFLC.PIV32TM and pFLC.PIV32TMcp45.
^d The numbers are the nt positions in the full-length antigenomic cDNA construct pFLC.PIV32CT and pFLC.PIV32CTcp45.

Sequence of pFLC.PIV32, 15492 bp in sense orientation
(only the insert is shown)

1 ACCAAACAAG AGAAGAACT TGTCTGGGAA TATAAATTTA ACTTTAAATT AACTTAGGAT
61 TAAAGACATT GACTAGAAGG TCAAGAAAAG GGAACCTCTAT AATTTCAAAA ATGTTGAGCC
121 TATTTGATAC ATTTAATGCA CGTAGGCAAG AAAACATAAC AAAATCAGCC GGTGGAGCTA
181 TCATTCTCTG ACAGAAAAAT ACTGTCTCTA TATTCGCCCT TGGACCGACA ATAAGTGATG
241 ATAATGAGAA AATGACATTA GCTCTTCTAT TTCTATCTCA TTCACTAGAT AATGAGAAAC
301 AACATGCACA AAGGGCAGGG TTCTTGGTGT CTTTATTGTC AATGGCTTAT GCCAATCCAG
361 AGCTCTACCT AACAAACAAAT GGAAGTAATG CAGATGTCAA GTATGTCATA TACATGATTG
421 AGAAAGATCT AAAACGGCAA AAGTATGGAG GATTTGTGGT TAAGACGAGA GAGATGATAT
481 ATGAAAAGAC AACTGATTGG ATATTTGGAA GTGACCTGGA TTATGATCAG GAAACTATGT
541 TGCAGAACGG CAGGAACAAT TCAACAATTG AAGACCTTGT CCACACATTT GGGTATCCAT
601 CATGTTTAGG AGCTCTTATA ATACAGATCT GGATAGTTCT GGTCAAAGCT ATCACTAGTA
661 TCTCAGGTTT AAGAAAAAGGC TTTTTCACCC GATTGGAAGC TTTTCAGACAA GTTGAACAG
721 TGCAGGCAGG GCTGGTATTG AGCGGTGACA CAGTGGATCA GATTGGGTCA ATCATGCGGT
781 CTCAACAGAG CTTGGTAACT CTTATGGTTG AAACATTAAT AACAATGAAT ACCAGCAGAA
841 ATGACCTCAC AACCATAGAA AAGAATATAC AAATTGTTGG CAACTACATA AGAGATGTCAG
901 GACCAAGCTT ATTCTTCAAT ACAATCAGAT ATGGAATTGA GACCAGAATG GCAGCTTTGA
961 CTCTATCCAC TCTCAGACCA GATATCAATA GATTAAAAGC TTTGATGGAA CTGTATTTAT
1021 CAAAGGGACC ACGCGCTCCT TTCATCTGTA TCCTCAGAGA TCCTATACAT GGTGAGTTCCG
1081 CACCAGGCAA CTATCCTGCC ATATGGAGCT ATGCAATGGG GGTGGCAGTT GTACAAAATA
1141 GAGCCATGCA ACAGTATGTG ACGGGAAGAT CATATCTAGA CATTGATATG TTCCAGCTAG
1201 GACCAAGCAGT AGCAGTGAT GCCGAAGCTC AAATGAGCTC AACACTGGAA GCGATCTTG
1261 GAGTGACACA CGAATCTAAA GAAAGCTTGA AGAGACATAT AAGGAACATA AACAGTTCAG
1321 AGACATCTTT CCACAAACCG ACAGGTGGAT CAGCCATAGA GATGGCAATA GATGAAGAGC
1381 CAGAACAATT CGAACATAGA GCAGATCAAG AACAAAATGG AGAACCTCAA TCATCCATAA
1441 TTCAATATGC CTGGGCAGAA GGAAATAGAA GCGATGATCA GACTGAGCAA GCTACAGAAT
1501 CTGACAATAT CAAGACCGAA CAACAAAACA TCAGAGACAG ACTAAACAAG AGACTCAACG
1561 ACAAGAAGAA ACAAGCAGT CAACCACCCA CTAATCCCAC AAACAGAACA AACCAGGACG
1621 AAATAGATGA TCTGTTTAAAC GCATTTGGAA GCAACTAATC GAATCAACAT TTTAATCTAA
1681 ATCAATAATA AATAAGAAAA ACTTAGGATT AAAGAATCCT ATCATACCGG AATATAGGGT
1741 GGTAAATTTA GAGTCTGCTT GAAACTCAAT CAATAGAGAG TTGATGGAAA CCGATGCTAA
1801 AAACATCAAA ATCATGGATT CTTGGGAAGA GGAATCAAGA GATAAATCAA CTAATATCTC
1861 CTCGGCCCTC AACATCATTG AATTCATACT CAGCACCGAC CCCCAGAAG ACTTATCGGA
1921 AAACGACACA ATCAACACAA GAACCCAGCA ACTCAGTGCC ACCATCTGTC AACCAGAAAT
1981 CAAACCAACA GAAACAAGTG AGAAAGATAG TGGATCAACT GACAAAAATA GACAGTCCGG
2041 GTCAATCACAC GAATGTACAA CAGAAGCAAA AGATAGAAAT ATTGATCAGG AAACGTACAC
2101 GAGAGGACCT GGGAGAAGAA GCAGCTCAGA TAGTAGAGCT GAGACTGTGG TCTCTGGAGG
2161 AATCCCCAGA AGCATCACAG ATTCTAAAAA TGGAAACCCAA AACACGGAGG ATATTGATCT
2221 CAATGAAATT AGAAAGATGG ATAAGGACTC TATTGAGGGG AAAATGCGAC AATCTGCAAA
2281 TGTTCCAAAGC GAGTATCAG GAAGTGATGA CATATTTACA ACAGAACAAA GTGGAACAG
2341 TGATCATGGA AGAAGCCTGG AATCTATCAG TACACCTGAT ACAAGATCAA TAAGTGTGTG
2401 TACTGCTGCA ACACCAGATG ATGAAGAAGA AATACTAATG AAAAATAGTA GGACAAAGAA
2461 AAGTTCTTCA ACACATCAAG AAGATGACAA AAGAATTAAA AAAGGGGGAA AAGGGAAAGA
2521 CTGGTTTAAG AAATCAAAAAG ATACCGACAA CCAGATACCA ACATCAGACT ACAGATCCAC
2581 ATCAAAAGGG CAGAAGAAAA TCTCAAAGAC AACCAACCACC AACACGGACA CAAAGGGGCA
2641 AACAGAAATA CAGACAGAAAT CATCAGAAAC ACAATCCTCA TCATGGAATC TCATCATCGA
2701 CAACAACACC GACCGGAACG AACAGACAAG CACAACCTCT CCAACAACAA CTTCCAGATC
2761 AACTTATACA AAAGAATCGA TCCGAACAAA CTCTGAATCC AAACCCAAGA CACAAAAGAC
2821 AAATGGAAAG GAAAGGAAGG ATACAGAAGA GAGCAATCGA TTTACAGAGA GGGCAATTAC
2881 TCTATTGCAG AATCTTGGTG TAATTCAATC CACATCAAAA CTAGATTTAT ATCAAGACAA
2941 ACGAGTTGTA TGTGTAGCAA ATGTACTAAA CAATGTAGAT ACTGCATCAA AGATAGATTT
3001 CCTGGCAGGA TTAGTCATAG GGGTTTCAAT GGACAACGAC ACAAAATTAA CACAGATACA
3061 AAATGAAATG CTAACCTCA AAGCAGATCT AAAGAAAATG GACGAATCAC ATAGAAGATT
3121 GATAGAAAAT CAAAGAGAAC AACTGTCATT GATCACGTCA CTAATTTCAA ATCTCAAAAT
3181 TATGACTGAG AGAGGAGGAA AGAAAGACCA AAATGAATCC AATGAGAGAG TATCCATGAT
3241 CAAAACAAAA TTGAAAGAAG AAAAGATCAA GAAGACCAGG TTTGACCCAC TTATGGAGGC
3301 ACAAGGCATT GACAAGAATA TACCCGATCT ATATCGACAT GCAGGAGATA CACTAGAGAA
3361 CGATGTACAA GTTAAATCAG AGATATTAAAG TTCATACAAAT GAGTCAAATG CACTCAAGAT
3421 AATACCCAAA AAAGTGAGCA GTACAATGAG ATCACTAGTT GCAGTCATCA ACAACAGCAA
3481 TCTCTACAAA AGCACAACAA AATCATACAT AAACGAACTC AAACGTTGCA AAAATGATGA
3541 AGAAGTATCT GAATTAATGG ACATGTTCAA TGAAGATGTC AACAAATGCC AATGATCCAA
3601 CAAAGAAACG ACACCGAACA AACAGAACAGT AGATCAAAAC CTGTCAACAC
3661 ACACAAAATC AAGCAGAATG AAAACAACAGA TATCAATCAA TATACAAATA AGAAAACTT
3721 AGGATTAAAG AATAAATTAA TCCTTGTTCA AAATGAGTAT AACTAACTCT GCAATATACA

TABLE 10 (cont'd)

3781	CATTCCCAGA	ATCATCATTG	TCTGAAAATG	GTCATATAGA	ACCATTACCA	CTCAAAGTCA
3841	ATGAACAGAG	GAAAGCAGTA	CCCCACATTA	GAGTTGCCAA	GATCGGAAAT	CCACCAAAAC
3901	ACGGATCCCG	GTATTTAGAT	GTCTTCTTAC	TCGGCTTCTT	CGAGATGGAA	CGAATCAAAG
3961	ACAAATACGG	GAGTGTGAAT	GATCTCGACA	GTGACCCGAG	TTACAAAGTT	TGTGGCTCTG
4021	GATCATTACC	AATCGGATTG	GCTAAGTACA	CTGGGAATGA	CCAGGAATTG	TTACAAGCCG
4081	CAACCAAAC	GGATATAGAA	GTGAGAAGAA	CAGTCAAAGC	GAAAGAGATG	GTTGTTTACA
4141	CGGTACAAAA	TATAAAACCA	GAACGTGTACC	CATGGTCCAA	TAGACTAAGA	AAAGGAATGC
4201	TGTTTCGATG	CAACAAAAGTT	GCTCTTGCTG	CTCAATGTCT	TCCACTAGAT	AGGAGCATAA
4261	AATTTAGAGT	AATCTTCGTG	AATTGTACGG	CAATTGGATC	AATAACCTTG	TTCAAAATTC
4321	CTAAGTCAAT	GGCATCACTA	TCTCTACCCA	ACACAATATC	AATCAATCTG	CAGGTACACA
4381	TAAAAACAGG	GGTTCAGACT	GATTCTAAAG	GGATAGTTCA	AATTTTGGAT	GAGAAAGGCG
4441	AAAAATCACT	GAAATTCATG	GTCCATCTCG	GATTGATCAA	AAGAAAAGTA	GGCAGAATGT
4501	ACTCTGTTGA	ATACTGTAAA	CAGAAAAATCG	AGAAAAATGAG	ATTGATATTT	TCTTTAGGAC
4561	TAGTTGGAGG	AATCAGTCTT	CATGTCAAATG	CAACTGGGTC	CATATCAAAA	ACACTAGCAA
4621	GTCAGCTGGT	ATTCAAAAAGA	GAGATTTGTT	ATCCTTTAAT	GGATCTAAAT	CCGCATCTCA
4681	ATCTAGTTAT	CTGGGCTTCA	TCAGTAGAGA	TTACAAGAGT	GGATGCAATT	TTCCAACCTT
4741	CTTTACCTGG	CGAGTTCAGA	TACTATCCTA	ATATTATTGC	AAAAGGAGTT	GGGAAAAATCA
4801	AACAATGGAA	CTAGTAATCT	CTATTTTAGT	CCGGACGTAT	CTATTAAGCC	GAAGCAAAAT
4861	AAGGATAATC	AAAAAATTAG	GACAAAAGAG	GTCAAATACCA	ACAACCTATTA	GCACTCACAC
4921	TCGCAAGAAT	AAGAGAGAAG	GGACCAAAAA	AGTCAAATAG	GAGAAATCAA	AACAAAAGGT
4981	ACAGAACACC	AGAACAACAA	AATCAAAACA	TCCAACCTCAC	TCAAAAACAAA	AATTCCAAAA
5041	GAGACCCGGG	ACACAACAAG	CACTGAACAC	CATGGATCAC	CTGCATCCAA	TGATAGTATG
5101	CATTTTTGTT	ATGTACACTG	GAATTTGAGG	TTCAGATGCC	ATTGCTGGAG	ATCAACTCCT
5161	CAATGTAGGG	GTCATTCAAT	CAAAGATAAG	ATCACTCATG	TACTACACTG	ATGGTGGCGC
5221	TAGCTTTTAT	GTTGTAAAT	TACTACCCAA	TCTTCCCCCA	AGCAATGGAA	CATGCAACAT
5281	CACCACTCTA	GATGCATATA	ATGTTACCCCT	ATTTAAGTTG	CTAACACCCC	TGATTGAGAA
5341	CCTGAGCAAA	ATTTCTGCTG	TTACAGATAC	CAAACCCCGC	CGAGAACGAT	TTGCAGGAGT
5401	CGTTATTGGG	CTTGCTGCAC	TAGGAGTAGC	TACAGCTGCA	CAAATAACCG	CAGCTGTAGC
5461	AATAGTAAAA	GCCAATGCAA	ATGCTGCTGC	GATAAACAAT	CTTGCACTCT	CAATTCAATC
5521	CACCAACAAG	GCAGTATCCG	ATGTGATAAC	TGCATCAAGA	ACAATTGCAA	CCGCAGTTCA
5581	AGCGATTTCAG	GATCACAATC	ATGGAGCCAT	TGTCAACGGG	ATAACATCTG	CATCATGCCG
5641	TGCCCATGAT	GCACTAATTG	GGTCAATATT	AAATTTGTAT	CTCACTGAGC	TTACTACAA
5701	ATTTTCATAAT	CAAATAACAA	ACCCTGCGCT	GACACCACTT	TCCATCCAAG	CTTTAAGAAT
5761	CCTCCTCGGT	AGCACCTTGC	CAATTGTCTAT	TGAATCCAAA	CTCAACACAA	AACTCAACAC
5821	AGCAGAGCTG	CTCAGTAGCG	GACTGTTAAT	TGGTCAAATA	ATTTCCATTT	CCCCAATGTA
5881	CATGCAATATG	CTAATTCAAA	TCAATGTTCC	GACATTTATA	ATGCAACCCG	GTGCGAAGGT
5941	AATTGATCTA	ATTGCTATCT	CTGCAAAACA	TAAATTACAA	GAAAGTAGTT	TACAAGTTCC
6001	TAATAGAAT	CTAGAATATG	CAAATGAACT	ACAAAACCTAC	CCAGCCAATG	ATTGTTTCTG
6061	GACACCAAAC	TCTGTATTTT	GTAGATACAA	TGAGGGTTCC	CCGATCCCTG	AATCACAATA
6121	TCAATGCTTA	AGGGGGAATC	TTAATTCTTG	CACTTTTACC	CCTATTATCG	GGAACCTTCT
6181	CAAGCGATTG	CACTTTGCCA	ATGGTGTCTC	CTATGCCAAC	TGCAAACTCT	TGCAATGTAA
6241	GTGTGCCGAC	CCTCCCCATG	TTGTGTCTCA	AGATGACAAC	CAAGGCATCA	GCATAATTGA
6301	TATTAAGAGG	TGCTCTGAGA	TGATGCTTGA	CACTTTTTC	TTTAGGATCA	CATCTACATT
6361	CAATGCTACA	TACGTGACAG	ACTTCTCAAT	GATTAATGCA	AATATTGTAC	ATCTAAGTCC
6421	TCTAGACTTG	TCAAATCAAA	TCAATTCAAT	AAACAAATCT	CTTAAAAGTG	CTGAGGATTG
6481	GATTGCAGAT	AGCAACTTCT	TCGCTAATCA	AGCCAGAACA	GCCAAGACAC	TTTATTCACT
6541	AAGTGCAATC	GCATTAATAC	TATCAGTGAT	TACTTTGGTT	GTTGTGGGAT	TGCTGATTGC
6601	CTACATCATC	AAGCTGGTTT	CTCAAATCCA	TCAATTGAGA	GCACTAGCTG	CTACAACAAT
6661	GTTCCACAGG	GAGAATCCTG	CCGTCTTTTC	CAAGAACAAT	CATGGAAACA	TATATGGGAT
6721	ATCTTAGGAT	CCCTACAGAT	CATTAGATAT	TAAAATTATA	AAAAACTTAG	GAGTAAAGTT
6781	ACGCAATCCA	ACTCTACTCA	TATAATTGAG	GAAGGACCCA	ATAGACAAAT	CCAAATCCAT
6841	GGAAGATTAC	AGCAATCTAT	CTCTTAAATC	AATTCCTAAA	AGGACATGTA	GAATCATTTT
6901	CCGAAGTGCC	ACAATTCTTG	GCATATGCAC	ATTAATTGTG	CTATGTTCAA	GTATTCTTCA
6961	TGAGATAATT	CATCTTGATG	TTTCTCTGCG	TCTTATGAAT	TCTGATGAGT	CACAGCAAGG
7021	CATTATTTCAG	CCTATCATAG	AATCATTAAA	ATCATTGATT	GCTTTGGCCA	ACCAGATTCT
7081	ATATAATGTT	GCAATAGTAA	TTCTCTTAA	AATTGACAGT	ATCGAAACTG	TAATACTCTC
7141	TGCTTTAAAA	GATATGCACA	CCGGGAGTAT	GTCCAATGCC	AACTGCACGC	CAGGAAATCT
7201	GCTTCTGCAT	GATGCAGCAT	ACATCAATGG	AAATAACAAA	TTCTTTGTAC	TTGAATCATA
7261	CAATGGGACG	CCTAAATATG	GACCTCTCCT	AAATATACCC	AGCTTTATCC	AGCTAGCAAC
7321	ATCTCCCCAT	GGGTGTACTA	GAATACCATC	ATTTTCACTC	ATCAAGACCC	ATTGGTGTTA
7381	CACTCACAAT	GTAATGCTTG	GAGATTGTCT	TGATTTACAG	GCATCTAACC	AGTATTTATC
7441	AATGGGGATA	ATACAACAAT	CTGCTGCAGG	GTTTCCAATT	TTCAGGACTA	TGAAAACCAT
7501	TTACCTAAGT	GATGGAATCA	ATCGCAAAAG	CTGTTCAAGT	ACTGCTATAC	CAGGAGGTTG
7561	TGCTTTGTAT	TGCTATGTAG	CTACAAGGTC	TGAAAAAGAA	GATTATGCCA	CGACTGATCT
7621	AGCTGAACTG	AGACTTGCTT	TCTATTATTA	TAATGATACC	TTTATTGAAA	GAGTCATATC

TABLE 10 (cont'd)

7681	TCTTCCAAAT	ACAACAGGGC	AGTGGGCCAC	AATCAACCCT	GCAGTCGGAA	GCGGGATCTA
7741	TCATCTAGGC	TTTATCTTAT	TTCTGTATA	TGGTGGTCTC	ATAAATGGGA	CTACTTCTTA
7801	CAATGAGCAG	TCCTCACGCT	ATTTTATCCC	AAAACATCCC	AACATAACTT	GTGCCGGTAA
7861	CTCCAGCAAA	CAGGCTGCAA	TAGCACGGAG	TTCTATGTCT	ATCCGTTATC	ACTCAAACAG
7921	GTTAATTCAG	AGTGCTGTTT	TTATTGTCTC	ATTGTCTGAC	ATGCATACAG	AAGAGTGTAA
7981	TCTAGTTATG	TTTAAACAAT	CCCAAGTCAT	GATGGGTGCA	GAAGGTAGGC	TCTATGTTAT
8041	TGGTAATAAT	TTGTATTATT	ATCAACGCAG	TTCTCTTGG	TGGTCTGCAT	CGCTCTTTTA
8101	CAGGATCAAT	ACAGATTTT	CTAAAGGAAT	TCTCCGATC	ATTGAGGCTC	AATGGGTACC
8161	GTCCTATCAA	GTTCTCTGTC	CTGGAGTCAT	GCCATGCAAT	GCAACAAGTT	TTTGCCCTGC
8221	TAATTGCATC	ACAGGGGTGT	ACGCAGATGT	GTGGCCGCTT	AATGATCCAG	AACTCATGTC
8281	ACGTAATGCT	CTGAACCCCA	ACTATCGATT	TGCTGGAGCC	TTTCTCAAAA	ATGAGTCCAA
8341	CCGAACATAAT	CCCACATTCT	ACACTGCATC	GGCTAACTCC	CTCTTAAATA	CTACCGGATT
8401	CAACAACACC	AATCACAAG	CAGCATATAC	ATCTTCAACC	TGCTTTAAAA	ACACTGGAAC
8461	CAAAAAAATT	TATTGTTTAA	TAATAATTGA	AATGGGCTCA	TCTCTTTTAG	GGGAGTTCCA
8521	AATAATACCA	TTTTTAAGGG	AACATAATGCT	TTAAGCTTAA	TTAACCATAA	TAAACCATAA
8581	TCTATCTATA	ATACAAGTAT	ATGATAAGTA	ATCTGCAATC	AGACAATAGA	CAAAAGGGAA
8641	ATATAAAAAA	CTTAGGAGCA	AAGCGTGCTC	GGGAAATGGA	CACTGAATCT	AACAATGGCA
8701	CTGTATCTGA	CATACTCTAT	CCTGAGTGTC	ACCTTAACTC	TCCTATCGTT	AAAGGTAAAA
8761	TAGCACAATT	ACACACTATT	ATGAGTCTAC	CTCAGCCTTA	TGATATGGAT	GACGACTCAA
8821	TACTAGTTAT	CACTAGACAG	AAAAATAAAC	TTAATAAATT	GGATAAAAAG	CAACGATCTA
8881	TTAGAAGATT	AAAATTAATA	TTAACTGAAA	AAGTGAATGA	CTTAGGAAAA	TACACATTTA
8941	TCAGATATCC	AGAAATGTCA	AAAGAAATGT	TCAAATTATA	TATACCTGGT	ATTAACAGTA
9001	AAGTAGCTGA	ATTATTACTT	AAAGCAGATA	GAACATATAG	TCAAATGACT	GATGGATTAA
9061	GAGTACTATG	GATTAATGTG	CTATCAAAAT	TAGCCTCAAA	AAATGATGGA	AGCAATTATG
9121	ATCTTAATGA	AGAAATTAAT	AATATATCGA	AAGTTCACAC	AACCTATAAA	TCAGATAAAT
9181	GGTATAATCC	ATTCAAAACA	TGGTTTACTA	TCAAGTATGA	TATGAGAAGA	TTACAAAAAG
9241	CTCGAAATGA	GATCACTTTT	AATGTTGGGA	AGGATTATAA	CTTGTTAGAA	GACCAGAAGA
9301	ATTTCTTATT	GATACATCCA	GAATTGGTTT	TGATATTAGA	TAAACAAAAC	TAAATGGTT
9361	ATCTAATTAC	TCCTGAATTA	GTATTGATGT	ATTGTGACGT	AGTCGAAGGC	CGATGGAATA
9421	TAAGTGCATG	TGCTAAGTTA	GATCCAAAAT	TACAATCTAT	GTATCAGAAA	GGTAATAACC
9481	TGTGGGAAGT	GATAGATAAA	TTGTTTCCAA	TTATGGGAGA	AAAGACATTT	GATGTGATAT
9541	CGTTATTAGA	ACCATTGCA	TTATCTTAA	TTCAAACCTA	TGATCCTGTT	AAACAACATA
9601	GAGGAGCTTT	TTTAAATCAT	GTGTTTCCG	AGATGGAATT	AATATTTGAA	TCTAGAGAAT
9661	CGATTAAGGA	ATTTCTGAGT	GATGATTACA	TTGATAAAAT	TTTAGATATA	TTTAATAAGT
9721	CTACAATAGA	TGAAATAGCA	GAGATTTTCT	CTTTTTTTAG	AACATTTGGG	CATCCTCCAT
9781	TAGAAGCTAG	TATTGCAGCA	GAAAAGGTTA	GAAAATATAT	GTATATTGGA	AAACAATTAA
9841	AATTTGACAC	TATTAATAAA	TGCTACTGTA	TCTTCTGTAC	AATAATAATT	AACGGATATA
9901	GAGAGAGGCA	TGGTGGACAG	TGGCCTCTG	TGACATTACC	TGATCATGCA	CACGATTACA
9961	TCATAAATGC	TTACGGTTCA	AACCTGCGA	TATCATATGA	AAATGCTGTT	GATTATTACC
10021	AGAGCTTTAT	AGGAATAAAA	TTCAATAAAT	TCATAGAGCC	TCAGTTAGAT	GAGGATTTGA
10081	CAATTTATAT	GAAAGATAAA	GCATTATCTC	CAAAAAATC	AAATTTGGGAC	ACAGTTTATC
10141	CTGCATCTAA	TTTACTGTAC	CGTACTAACG	CATCCAACGA	ATCAGGAAGA	TTAGTTGAAG
10201	TATTTATAGC	AGATAGTAAA	TTTGATCCTC	ATCAGATATT	GGATTATGTA	GAATCTGGGG
10261	ACTGGTTAGA	TGATCCAGAA	TTTAATATTT	CTTATAGTCT	TAAAGAAAAA	GAGATCAAC
10321	AGGAAGGTAG	ACTCTTTGCA	AAAATGACAT	ACAAAATGAG	AGCTACACAA	GTTTTATCAG
10381	AGACCTACT	TGCAATAAAC	ATAGGAAAA	TCTTTCAAGA	AAATGGGATG	GTGAAGGGAG
10441	AGATTGAATT	ACTTAAGAGA	TTAACAACCA	TATCAATATC	AGGAGTTCCA	CGGTATAATG
10501	AAGTGTACAA	TAATTCTAAA	AGCCATACAG	ATGACCTTAA	AACCTACAAT	AAAATAAGTA
10561	ATCTTAATTT	GTCTTCTAAT	CAGAAATCAA	AGAAATTTGA	ATTCAAGTCA	ACGGATATCT
10621	ACAATGATGG	ATACGAGACT	GTGAGCTGTT	TCCTAACAAC	AGATCTCAAA	AAATACTGTC
10681	TTAATTGGAG	ATATGAATCA	ACAGCTCTAT	TTGGAGAAAC	TTGCAACCAA	ATATTTGGAT
10741	TAAATAAATT	GTTTAATTGG	TTACACCTC	GTCTTGAAGG	AAGTACAATC	TATGTAGGTG
10801	ATCCTTACTG	TCCTCCATCA	GATAAAGAAC	ATATATCATT	AGAGGATCAC	CCTGATTCTG
10861	GTTTTTACGT	TCATAACCCA	AGAGGGGGTA	TAGAAGGATT	TTGTCAAAAA	TTATGGACAC
10921	TGATCTCTAT	AAGTGAATA	CATCTAGCAG	CTGTTAGAAT	AGGCGTGAGG	GTGACTGCAAT
10981	TGTTTCAAGG	AGACAATCAA	GCTATAGCTG	TAAACACAAG	AGTACCCAAC	AATTGACT
11041	ACAGAGTTAA	GAAGGAGATA	GTTTATAAAG	ATGTAGTGAG	ATTTTTTGAT	TCATTAAGAG
11101	AAGTGTATGA	TGATCTAGGT	CATGAACCTA	AATTAATGA	AACGATTATA	AGTAGCAAGA
11161	TGTTTCATATA	TAGCAAAAAG	ATCTATTATG	ATGGGAGAAT	TCTTCTCAA	GCTCTAAAAG
11221	CATTATCTAG	ATGTGTCTTC	TGGTCAGAGA	CAGTAATAGA	CGAAACAAGA	TCAGCATCTT
11281	CAAATTTGGC	AACATCATTT	GCAAAAGCAA	TTGAGAATGG	TTATTCACCT	GTTCTAGGAT
11341	ATGCATGCTC	AATTTTTAAG	AATATTCAAC	AACTATATAT	TGCCCTTGGG	ATGAATATCA
11401	ATCCAACCTAT	AACACAGAAT	ATCAGAGATC	AGTATTTTAG	GAATCCAAAT	TGGATGCAAT
11461	ATGCCTCTTT	AATACCTGCT	AGTGTGGGG	GATTCAATTA	CATGGCCATG	TCAAGATGTT
11521	TTGTAAGGAA	TATTGGTGAT	CCATCAGTTG	CCGCATTGGC	TGATATTAAA	AGATTATTATA

TABLE 10 (cont'd)

11581	AGGCGAATCT	ATTAGACCGA	AGTGTCTTT	ATAGGATTAT	GAATCAAGAA	CCAGGTGAGT
11641	CATCTTTTTT	GGACTGGGCT	TCAGATCCAT	ATTTCATGCAA	TTTACCACAA	TCTCAAAATA
11701	TAACCAACCAT	GATAAAAAAT	ATAACAGCAA	GGAATGTATT	ACAAGATTCA	CCAAATCCAT
11761	TATTATCTGG	ATTATTCACA	AATACAATGA	TAGAAGAAGA	TGAAGAATTA	GCTGAGTTCC
11821	TGATGGACAG	GAAGGTAATT	CTCCCCTAGAG	TTGCACATGA	TATTCTAGAT	AATTCTCTCA
11881	CAGGAATTAG	AAATGCCATA	GCTGGAATGT	TAGATACGAC	AAAATCACTA	ATTCGGGTTG
11941	GCATAAATAG	AGGAGGACTG	ACATATAGTT	TGTTGAGGAA	AATCAGTAAT	TACGATCTAG
12001	TACAATATGA	AACACTAAGT	AGGACTTTGC	GACTAATTGT	AAGTGATAAA	ATCAAGTATG
12061	AAGATATGTG	TTCGGTAGAC	CTTGCCATAG	CATTGCGACA	AAAGATGTGG	ATTTCATTTAT
12121	CAGGAGGAAG	GATGATAAGT	GGACTTGAAA	CGCCTGACCC	ATTAGAAATTA	CTATCTGGGG
12181	TAGTAATAAC	AGGATCAGAA	CATTGTAAAA	TATGTTATTC	TTCAGATGGC	ACAAACCCAT
12241	ATACTTGGAT	GTATTTACCC	GGTAATATCA	AAATAGGATC	AGCAGAAACA	GGTATATCGT
12301	CATTAAGAGT	TCCTTATTTT	GGATCAGTCA	CTGATGAAAG	ATCTGAAGCA	CAATTAGGAT
12361	ATTCAAGAA	TCTTAGTAAA	CCTGCAAAAG	CCGCAATAAG	AATAGCAATG	ATATATACAT
12421	GGGCATTGGG	TAATCTTGAG	ATATCTTGGG	TGGAAGCCTC	ACAGATAGCA	CAACACCGTG
12481	CAAAATTTTAC	ACTAGATAGT	CTCAAAATTT	TAACACCGGT	AGCTACATCA	ACAAATTTTAT
12541	CACACAGATT	AAAGGTAAC	GCAACTCAGA	TGAAATCTC	CAGTACATCA	TTGATCAGAG
12601	TCAGCAGATT	CATAACAATG	TCCAATGATA	ACATGTCTAT	CAAAGAAGCT	AATGAAACCA
12661	AAGATACTAA	TCTTATTTAT	CAACAATAA	TGTTAACAGG	ATTAAGTGT	TTCGAATATT
12721	TATTTAGATT	AAAAGAAACC	ACAGGACACA	ACCCATAGT	TATGCATCTG	CACATAGAAG
12781	ATGAGTGTG	TATTAAGAA	AGTTTTAATG	ATGAACATAT	TAATCCAGAG	TCTACATTAG
12841	AATTAATTCG	ATATCCTGAA	AGTAATGAAT	TTATTTATGA	TAAAGACCCA	CTCAAAGATG
12901	TGGACTTATC	AAAACCTATG	GTTATTAAG	ACCATTCTTA	CACAATTGAT	ATGAATTATT
12961	GGGATGCATC	TGACATCATA	CATGCAATTT	CAATATGTAC	TGCAATTACA	ATGCAAGATA
13021	CTATGTCACA	ATTAGATCGA	GATAATTTAA	AAGAGATAAT	AGTTATTGCA	AATGATGATG
13081	ATATTAATAG	CTTAATCACT	GAATTTTTGA	CTCTTGACAT	ACTTGATTTT	CTCAAGACAT
13141	TTGGTGGATT	ATTAGTAAAT	CAATTTGCAT	ACACTCTTTA	TAGTCTAAAA	ATAGAAGGTA
13201	GGGATCTCAT	TTGGGATTAT	ATAATGAGAA	CACTGAGAGA	TACTTCCCAT	TCAATATTAA
13261	AAGTATTATC	TAATGCATTA	TCTCATCCTA	AAGTATTCAA	GAGGTTCTGG	GATTGTGGAG
13321	TTTTAAACCC	TATTTATGGT	CCTAATACTG	CTAGTCAAGA	CCAGATAAAA	CTTGCCCTAT
13381	CTATATGTGA	ATATTCACCTA	GATCTATTTA	TGAGAGAATG	GTTGAATGGT	GTATCACTTG
13441	AAATATACAT	TTGTGACAGC	GATATGGAAG	TTGCAAAATGA	TAGGAAACAA	GCCTTTATTT
13501	CTAGACACCT	TTCATTTGTT	TGTTGTTTTAG	CAGAAATTGC	ATCTTTTCGGA	CCTAACCTGT
13561	TAAACTTAAC	ATACTTGGAG	AGACTTGATC	TATTGAAACA	ATATCTTGAA	TTAAATATTA
13621	AAGAAGACCC	TACTCTTAAA	TATGTACAAA	TATCTGGATT	ATTAATTTAA	TCGTTCCCAT
13681	CAACTGTAAC	ATACGTAAGA	AAGACTGCAA	TCAAATATCT	AAGGATTCCG	GGTATTAGTC
13741	CACCTGAGGT	AATTGATGAT	TGGGATCCGG	TAGAAGATGA	AAATATGCTG	GATAACATTG
13801	TCAAAACTAT	AAATGATAAC	TGTAATAAAG	ATAATAAAGG	GAATAAAATT	AACAAATTTCT
13861	GGGACTAGC	ACTTAAGAAC	TATCAAGTCC	TTAAAATCAG	ATCTATAACA	AGTGATTCTG
13921	ATGATAATGA	TAGACTAGAT	GCTAATACAA	GTGGTTTGAC	ACTTCCCTCA	GGAGGGAATT
13981	ATCTATCGCA	TCAATTGAGA	TTATTCGGAA	TCAACAGCAC	TAGTTGTCTG	AAAGCTCTTG
14041	AGTTATCACA	AATTTTAATG	AAGGAAGTCA	ATAAAGACAA	GGACAGGCTC	TTCTCTGGAG
14101	AAGGAGCAGG	AGCTATGCTA	GCATGTTATG	ATGCCACATT	AGGACCTGCA	GTTAATTATT
14161	ATAATTCAGG	TTTGAATATA	ACAGATGTAA	TTGGTCAACG	AGAATTGAAA	ATATTTCCCT
14221	CAGAGGTATC	ATTAGTAGGT	AAAAAATTAG	GAAATGTGAC	ACAGATTCTT	AACAGGGTAA
14281	AAGTACTGTT	CAATGGGAAT	CCTAATTCAA	CATGGATAGG	AAATATGGAA	TGTGAGAGCT
14341	TAATATGGAG	TGAATTAAT	GATAAGTCCA	TTGGATTAGT	ACATTGTGAT	ATGGAAGGAG
14401	CTATCGGTAA	ATCAGAAGAA	ACTGTTCTAC	ATGAACATTA	TAGTGTATA	AGAATTACAT
14461	ACTTGATTGG	GGATGATGAT	GTTGTTTTAG	TTTCCAAAAT	TATACCTACA	ATCACTCCGA
14521	ATTGGTCTAG	AATACTTTAT	CTATATAAAT	TATATTGGAA	AGATGTAAGT	ATAATATCAC
14581	TCAAAACTTC	TAATCCTGCA	TCAACAGAAAT	TATATCTAAT	TTCGAAAGAT	GCAATTTGTA
14641	CTATAATGGA	ACCTAGTGAA	ATTGTTTTAT	CAAAACTTAA	AAGATTGTCA	CTCTTGGAAG
14701	AAAATAATCT	ATTAATAATG	ATCATTTTAT	CAAAGAAGAG	GAATAATGAA	TGGTTACATC
14761	ATGAAATCAA	AGAAGGAGAA	AGAGATTATG	GAATCATGAG	ACCATATCAT	ATGGCACTAC
14821	AAATCTTTGG	ATTTCAAATC	AATTTAAATC	ATCTGGCGAA	AGAATTTTAA	TCAACCCGAG
14881	ATCTGACTAA	TATCAACAAT	ATAATCCAAA	GTTTTACAGC	AACAATAAAG	GATGTTTTAT
14941	TTGAATGGAT	TAATATAACT	CATGATGATA	AGAGACATAA	ATTAGGCGGA	AGATATAACA
15001	TATTTCCACT	GAAAAATAAG	GGAAAGTTAA	GACTGCTATC	GAGAAGACTA	GTATTAAGTT
15061	GGATTTTCAT	ATCATTATCG	ACTCGATTAC	TTACAGGTCG	CTTTCCTGAT	GAAAAATTTG
15121	AACATAGAGC	ACAGACTGGA	TATGTATCAT	TAGCTGATAC	TGATTTAGAA	TGATTTAGAA
15181	TATTGTGCGA	AAACATCATT	AAGAATTACA	GAGAGTGTAT	AGGATCAATA	TCATATTGGT
15241	TTCTAACCAA	AGAAGTTAAA	ATACTTATGA	AATTGATCGG	TGGTGCTAAA	TTATTAGGAA
15301	TTCCAGAGCA	ATATAAAGAA	CCCGAAGACC	AGTTATTAGA	AAACTACAAT	CAACATGATG
15361	AATTTGATAT	CGATTAATAA	ATAAATACAA	TGAAGATATA	TCCTAACCTT	TATCTTTAAG
15421	CCTAGGAATA	GACAAAAAAGT	AAGAAAAACA	TGTAATATAT	ATATACCAAA	CAGAGTTCTT

15481 CTCTTGTTTG GT

Sub. a/3

In a second strategy (Figure 7), chimeric PIV3-PIV2 F and HN ORFs rather than the complete ORF exchange were constructed in which regions of the PIV2 F and HN ORFs encoding the ectodomains were amplified from pLit.PIV32Fhc and pLit.PIV32HNhc, respectively, using PCR, Vent DNA polymerase (NEB, Beverly, MA), and primer pairs specific to PIV2 F (5, 6 in Table 9) and HN (7, 8 in Table 9). In parallel, the regions of PIV3 F and HN ORFs encoding the ectodomains were deleted from their cDNA subclones pLit.PIV3.F3a and pLit.PIV3.HN4 (Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference), respectively, using PCR, Vent DNA polymerase, and primer pairs specific to PIV3 F (9, 10 in Table 9) and HN (11, 12 in Table 9). The amplified F and HN cDNA fragments of PIV2 and PIV3 were purified from agarose gels and ligated to generate pLit.PIV32FTM and pLit.PIV32HNTM, respectively. The chimeric F and HN constructs were digested with PpuMI plus SpeI and assembled together to generate pLit.PIV32TM, which was subsequently sequenced with the dRhodamine dye terminator sequencing kit across its PIV specific region in its entirety and found to be as designed. The 4 kb BspEI-SpeI fragment from pLit.PIV32TM was then introduced into the BspEI-SpeI window of p38'ΔPIV31hc to generate p38'ΔPIV32TM. The 6.5 kb BspEI-SphI fragment from p38'ΔPIV32TM, containing the PIV3-PIV2 chimeric F and HN genes, was introduced into the BspEI-SphI window of pFLC.2G+.hc and pFLCcp45 (Skiadopoulos et al., *J. Virol.* 73:1374-81, 1999, incorporated herein by reference) to generate pFLC.PIV32TM (Table 11; SEQ ID NO:) and pFLC.PIV32TMcp45, respectively. The nucleotide sequence of the BspEI-SpeI fragment, containing the chimeric PIV3-PIV2 F and HN genes, is submitted in the GenBank (Accession No. pending).

Sub 9/4

TABLE 11 (SEQ ID NO: 1)

Sequence of pFLC.PIV32TM, 15498 bp in sense orientation
(only the antigenome is shown)

1 ACCAAACAG AGAAGAACT TGCTGGGAA TATTAATTA ACTTAAATT AACTTAGAT
61 TAAAGACATT GACTAGAGG TCAAGAAAAG GGAACCTTAT AATTTCAAA ATGTTGAGCC
121 TATTGTATC ATTTATGCA CGTAGGCAAG AAAACATAAC AAATCAGCC GGTGAGCTA
181 TCATTCCCTG ACAGAAAAT ACTGCTCTTA TATTCGCCCT TCGACCGACA ATPACTGATG
241 ATATAGAGAA AATGACATTA GCTCTTCTAT TTCTATCTCA TTCACCTAGT AATGAGAAAC
301 AACATGCACA AAGGCGAGG TTCTTGCTGT CTTTATGTTC AATGCTTAT GCCAATCCAG
361 AGCTTACTCT AACACAAAT GGAAGTAATG CAGATGTCAA GTATGTCTA TACATGATATG
421 AGAAGATCT AAAACGGCA AAGTATGAG GATTTGTGT TAAGCGAGA GAGATGATAT
481 ATGAAAAGAC AACTGATGG ATATTGGAA GTGACCTGGA TTATGATCAG GAAACTATGT
541 TGCAGACGG CAGGACAT TCACCAATG AAGACCTGT CCACACATTT GGTATCCAT
601 CATGTTTAGG AGCTCTTATA ATACAGATCT GATAGTTCT GGTCAAAGCT ATCACTAGTA
661 TCTCAGGCTT AAGAAAAGC TTTTCACCC GATTGGAAG CATTGGTCA ATCATGCCG
721 TGCAGGAGG GCTGTATG AGCGTGACA CAGTGATCA GATTGGTCA ATCATGCCG
781 CTCAACAGAG CTGTGTAAT CTTATGTTG AAACATTAAT AACATGAAT ACCAGCAGAA
841 ATGACCTCAC AACCATAGAA AAGATATATC AAATTTGTG CAACTACATA AGAGATCAG
901 GTCTCGCTC ATCTTCAAT ACAATCAGAT ATGGAATTGA GACCAGAATG GCAGCTTGA
961 CTCTATCCAC TCTCAGACA GATATCAATA GATTAAAGC TTTGATGAA CTGTAATTAT
1021 CAAAGGGACC ACGGCTCTT TTCATCTGTA TCCTCAGAGA TCCTATACAT GGTGAGTTG
1081 CACCAGGCA CTATCTGCC ATATGAGAGT ATGCAATGGG GTTGCAGT TTCCAAATA
1141 GAGCATGCA ACAGTATG ACGGAAGAT CATATCTAGA CATGTATG TTCCAGTAG
1201 GACAAAGAGT AGCAGTGAT GCCGAAGCTC AAATGAGCTC AACCTGGA GATGAAGTTG
1261 GAGTGACAC CGAATCTTAA GAAAGCTTGA AGAGACATAT AAGGAACATA AACAGTTGAG
1321 AGACATCTT CCACAAACC ACAGTGGAT CAGCCATAGA GATGCCATA GATGAAGAGC
1381 CAGAACATTT CGAACATAGA GCAGATCAAG AACAAATGG AGAACCTCAA TCATCCATTA
1441 TTCAATATGC CTGGCGAGAA GGAATAGAA CGCATGATCA GACTGAGCAA GCTACAGAA
1501 CTGACATAT CAAGACCGA CAACAAACA TCAGAGACAG ACTAAACAG AGACTCAAG
1561 ACAAGAGAA ACAAGAGAT CAACACCCA CTAATCCAC AACACAGACA ACCAGAGC
1621 AATATGATGA TCTGTTTAC GCATTGGA GCACTAATC GAATCAACAT TTTAATCTAA
1681 ATCAATATA AATAAGAAA ACTTAGATTT AAAGAATCT ATCATACCG AATAATAGGT
1741 GATAATTTA GAGTCTGCT GAACTCAAT CATATAGAG TTGATGAAA GCGATGCTAA
1801 AAATATCAA ATCATGATTT CTGGGAGA GGAATCAGA GATTAATCAA CTATATCTC
1861 CTGGGCTCT AACATCATG AATTCATACT CAGCACCGAC CCCCAAGAG ACTTATCGA
1921 AAGCAGACA ATCAACAGA GAACCCAGA ACTCAGTGC ACCATCTGT GACCAATAT
1981 CAAACACACA GAAACAAGT AGAAGATAG TGGATCACT GACAAAATA AACTGCCG
2041 GTCAATCAC GAATGTACA CAGAGCAAA AGATGANAAT ATGATCAGG AAATGTACA
2101 GAGAGGACT GGGAGAGAA GCAGCTCAGA TAGTAGAGT GAGACTGTG TCTCTGAGG
2161 AATCCCGA AGCATCACG ATTCTAANA TGAACCCAA AACACGGAG ATATTGATCT
2221 CAATGAATTT AGAAGATGG ATAGGACTC TATTTAGGG AAAATGCGA AATCTGCAA
2281 TGTTCACAG GAGATATCAG GAATGATGA CATATTYACA ACAGAACAA GTAGAAACAG
2341 TGATCATGA AGAGCTGG AATCTATGAT TACACCTGAT AAAATAGTA GACGTGTGT
2401 TACTGCTGCA ACACAGATG ATGAAGAGA AATACTAATG AAAATAGTA GACGTGTGT
2461 AAGTTCTTCA ACACATCAG AAGATGACAA AAGAATTAA AAAGGGGAA AAGGGAAGA
2521 CTGTTTAG AAATCAAAG ATACCGACA CCAGATACCA ACATCAGACT ACAGATCCAC
2581 ATCAAAGGG CAGAGAANA TCTCAAAGC AACACACAC AACACGACA CAAAGGGCA
2641 AACAGAAATA CAGACAGAT CATCAGAAAC ACAATCTCA TCATGGAATC TCATCATGA
2701 CAACACACC GACCGAGC AACAGACAA CACAATCTT CCACACAGA CTTCACATG
2761 AACTATATCA AAGAATCGA TCCGACAAA CTCTGATCC AAACCCAGA CACCAAGATC
2824 AATGGAAG GAAAGGAGG ATAGAGAGA GAGCAATCGA TTTACAGAGA GGGCAATTAC
2881 TCTATTGCGG AATCTTGTG TATTTCAATC CACATCAAAA CTAGATTAT ATCAAGACAA

TABLE 11 (cont'd)

3721	AGGATTAAG	AATAATTAA	TCCTGTCCA	AAATGAGTAT	AACTAAGTCT	GCAATATACA
3781	CATCCCAGA	ATCATCATTC	TCGTAAATG	GTCAATATAGA	ACCATTAACA	CTCAAGTCA
3841	ATGAACAGAG	GAAGCAGTA	GAGTTGCCAA	GATCGAAT	CCACCAAAAC	
3901	ACGGATCCCG	GTATTAGAT	GCTTCTTAC	TCGGCTCTT	CGAGATGGAA	CGAATCAAG
3961	ACAATACCG	GAGTGAAT	GATCTGACA	GTGACCCGAG	TTACAAAGTT	TGTGGCTCTG
4021	GATCATACC	AATCGATTG	GCTAGTACA	CTGGGAATGA	CCAGGAATG	TTACAGCCG
4081	CAACCAACT	GGATATAGAA	GTCGAGAA	CAGTCAAGC	GAAAGAGATG	GTTGTTTACA
4141	CGTACAAA	TATAAACCA	GAACTGTACC	CATGTCCTCA	TAGACTAAGA	AAAGAAATGC
4201	TGTTGATGC	CAACAAAGTT	GCTCTTGCTG	CTCAATGCTC	TCCACTAGAT	AGAGCAATTA
4261	AATTTAGAT	AATCTTCGTG	AATTTGATCG	CAATTTGATC	AATAACCTTG	TTCAAAATTC
4321	CTAAGTCAAT	GGCATCAGTA	TCTCTACCA	ACACAATATC	AATCAATCTG	CAGGTACACA
4381	TAAAAACAGG	GGTTCAGACT	GATTTCTAAG	GGATAGTTCA	AAITTTGGAT	GAGAAAGCG
4441	AAAATCACT	GAATTTCATG	GTCCATCTCG	GATTGATCAA	AAGAAAAGTA	GGCAGATGT
4501	ACTCTGTGA	ATACTGTAA	CAGAAATCG	AGAAAATGAG	ATGATATTTT	TCTTTAGGAC
4561	TAGTTGAGG	AATCAGTCTT	CATGTCATG	CAACTGGGTC	CATATCAAAA	ACACTAGCAA
4621	GTGAGCTGGT	ATTCAAAAGA	GAGATTTGTT	ATCCTTTAAT	GGATCTAAT	CCGCATCTCA
4681	ATCTAGTTAT	CTGGCTTCA	TCAGTAGAGA	TTACAAGAGT	GGATGCAATT	TTCCAACTT
4741	CTTACCTGG	CGAGTTCAGA	TACTATCTTA	ATATTATTGC	AAAAGAGATT	GGGAAAATCA
4801	AACAATGGAA	CTAGTAATCT	CTATTTAGT	CCGACGCTAT	CTATTTAAGC	GAAGCAATA
4861	AAGGATAATC	AAAACTTAG	GACAAAAGAG	GTCAATACCA	ACAACTATTA	GCAGTCACAC
4921	TCGCAAGAT	AAGAGAGAA	GGACCAAAA	AGTCAATAG	GAGAAATCAA	AACAAAAGT
4981	ACAGAACACC	AGAACAACA	AATCAAAACA	TCCAATCAC	TCAAAACAAA	AAITTCAAA
5041	GAGACCGCA	ACACACACAG	CACAGACATG	GCATCACTG	TCAGCAATGA	TAATTCGAA
5101	TTTGTATG	TACACTGGAA	TTGTAGGTC	AGATGCCATT	GCTGGAGATC	AACTCTCAA
5161	TGTAGGGTC	ATCAATCAA	AGATAAGATC	ACTCATGTAC	TACACTGATG	GTGGCGCTAG
5221	CTTTATGTT	GTAATATTAC	TACCCAATCT	TCCCCCAAGC	AATGGAACAT	GCAACATCAC
5281	CAGCTAGAT	GCATATTAAG	TTACCTTATT	TAAGTGCTA	ACACCCCTGA	TTGAGAACTT
5341	GAGCAAAAT	TCTGCTGTTA	CAGATACCAA	ACCCTGCCGA	GAAGCAATTG	CAGAGTCTGT
5401	TATTTGGCTT	GCTGCACATG	GAGTAGCTAC	AGCTGCACAA	ATAACCCGCA	CTGTATCCAC
5461	AGTAAAGCC	AATGCAATAG	CTGCTGCCAT	AAACAATCTT	GCATCTTCAA	TTCAATCCAC
5521	CAACAAGGCA	GTATCCGATG	TGATTAAGTGC	ATCAAGACCA	ATTCGAACCG	CAGTTCAAGC
5581	GATTCAGGAT	CACATCAATG	GACCCATTGT	CAACGGGATA	ACATCTGCAT	CATGCCGTGC
5641	CCATGATGCA	CTAATTGGGT	CAATATTAAA	TTTGTATCTC	ACTGAGCTTA	CTACATATT
5701	TCATTAATCAA	ATAACAACCC	CTGCGTGCAC	ACCACCTTCC	AATCAAGCTT	TAAAGATTCCT
5761	CCTCGGTAGC	ACCTGGCCAA	TTGTCAATGA	ATCCAACATC	AATCAAAAAC	TCACACAGC
5821	AGAGCTGCTC	AGTAGCGGAC	TGTTAAGTGG	TCAATAATTT	TCCATTTCCC	CAATGATAC
5881	GCAATGCTA	ATTCAATCA	ATGTTCCGAC	ATTATTAATG	CAACCCGGTG	CGAAGCTAAT
5941	TGATCTAAT	GCTATCTCTG	CAAACCATAA	ATTACAGAA	GTAGTTGTAC	AAAGTTCTTAA
6001	TAGATTTCTA	GAATATGCAA	ATGAATACCA	AAACTACCCA	GGCAATGATT	GTTTCGTGAC
6061	ACCAACTCT	GTATTTTCTA	GATPACATGA	GGGTTCCCGG	ATCCCTGAAT	CACATATACA
6121	ATGCTTAAGG	GGGAATCTTA	ATCTTGACAC	TTTAAACCTT	ATTATCGGGA	ACTTTCTCAA
6181	GGGATTCGCA	TTTGCCCATG	GTGTCTCTA	TGCCAATGCG	AAATCTTTGC	TATGTAGATG
6241	TGCCGACCC	CCCCATGTTG	TGCTCAAGA	TGACAACCAA	GGCATCAGCA	TAAITGATAT
6301	TAAAGAGTGC	TCTGAGATGA	TGCTTGACAC	TTTTTCATTT	AGGATCACAT	CTACATTTCAA
6361	TGCTACATAC	GTGACAGACT	TCTCAATGAT	TAAITGCAAT	ATTGTACATC	TAACTCTCT
6421	AGACTGTCTA	AATCAATCA	ATTCATATA	CAATCTCTT	AAAAGTCTG	AGGATTTGAT
6481	TGCAGATAGC	AACTTCTTCG	CTAATCAAGC	CAGAACAGCC	AAGACACTTT	ATTCACTAAT
6541	CATAATTATT	TTGATAATGA	TCAITTAAT	GTTTATTAAT	AATATAACGA	TAAITTAAT
6601	TGCAATTAG	TATTACAGAA	TTCAAAAGAG	AAATCGAGTG	GATCAAAATG	ACAAAGCAATA
6661	TGTACTAACA	AACAATTAAC	ATATCTAGAT	ATCAATTAAT	ATTAAATTA	TAAAAAACCT
6721	AGGAGTAAAG	TTAGCAATC	CACTCTACT	CATATTAATG	AGGAAGGACC	CATAGACAAA
6781	ATGCAATTC	GAGATGCAAT	TTTGTAGCA	TACCAATCAC	GGAAAGGATG	CTGGTAAATGA

TABLE 11 (cont'd)

7621	TCTAGCTGAA	CTGAGACTTG	CTTCTATTTA	TTATATGAT	ACCTTTATTG	AAAGACTCAT
7681	ATCTCTTCCA	AATACACAG	GGCAGTGGCC	CACAATCAAC	CCTGCAGTCG	GAAGCGGAT
7741	CTATCATCTA	GGCTTATCT	TATTTCTCTG	ATATGGTGGT	CTCATTAATG	GGACTACTTC
7801	TTACAAATGAG	CAGTCCCTAC	GCTATTTTAT	CCCAAAACAT	CCCACATTA	CTTGTCGGG
7861	TAACCTCCAGC	AAACAGGCTG	CAATAGCAGC	GAGTTCCTAT	GTCATCCGTT	ATCACTCAAA
7921	CAGGTTAATT	CAGAGTGGTG	TTCTTATTTG	TCCATTTGCT	GACATGCATA	CAGAAAGAGTG
7981	TAATCTAGTT	ATGTTTACA	ATTCOCAAGT	CATGATGGGT	GCAGAAGTA	GGCTCTATGT
8041	TATTTGTAAT	AATTGTAAT	ATTATCAACG	CAGTTCCTCT	TGGTGGCTG	CATGCTCTT
8101	TTACAGAGTC	AATACAGATT	TTTCTAAAG	AATTCCTCCG	ATCATTGAGG	CTCATATATG
8161	ACCGTCCAT	CAGTTCCTC	GTCCTGAGG	CATGCCATGC	AATGCACAAA	GTTTTTGCCC
8221	TGCTAATTC	ATCACAGGG	TGTACGCGA	TGTGTGGCCG	CTTAATGATC	CAGAACTCAT
8281	GTCAGTAAT	GCTCTGAAC	CCAACTATCG	ATTGCTGGA	GCCTTTCTCA	AAAATGAGTC
8341	CAACCGAAT	AATCCCAT	TCTACACTGC	ATCGGCTAAC	TCCCTCTTAA	ATACTACCGG
8401	ATTCAACAAC	ACCAATCACA	AAGCAGCATTA	TACATCTTCA	ACCTGCTTTA	AAAACACTGG
8461	AACCCAAAAA	ATTATTTGTT	TAATTAATAT	TGAATGGGC	TCATCTCTTT	TAGGGGAGTT
8521	CCAATTAATA	CCATTTTAA	GGGAATTAAT	GCTTTAAGT	TCATTAATTA	CCATATATATG
8581	CATCAATCTA	TCTATATATC	AAGTATATGA	TAAGTAATCA	GCAATCAGAC	AATAGACAAA
8641	AGGGAATAT	AAAAAATTA	GGAGCAAAAG	GTCCTGGGA	AATGACACAT	GAATCTTAACA
8701	ATGGCACTGT	ATCTGACATA	CTCTATCTTG	AGTGTACCT	TAACCTCTCT	ATCGTTAAAG
8761	GTAATAATAGC	ACAATTAAC	ACTATTAATGA	GCTTACCTCA	GCCTTATGAT	ATGGATGACG
8821	ACTCAATACT	AGTTATCACT	AGACAGAAA	TAAAACTTAA	TAAATTTGAT	AAAAGACAAC
8881	GATCTATTAG	AAGATTAAAA	TTAATTAATTA	CTGAAAAGT	GAATGACTTA	GGAATATACA
8941	CATTATCAG	ATATCCAGAA	ATGTCAAAG	AAATGTCAA	ATTATATATA	CTCGTATTA
9001	ACAGTAAAGT	GACTGAATTA	TTACTTAAAG	CAGATAGAAC	ATATAGTCAA	ATGACTGATG
9061	GATTAGAGAG	TCTATGGAAT	AATGTGCTAT	CAAAATTAGC	CTCAAAAAT	GATGGAAGCA
9121	ATTATGATCT	TAATGAGAA	ATTAATATA	TATCGAAAGT	TCACACAACC	TATTAATTCAG
9181	ATAAATGGTA	TAATCCATTC	AAAACATGCT	TTACTATCAA	GTATGATATG	AGAAGATTAC
9241	AAAAAGCTCG	AAATGAGATC	ACTTTTAATG	TTGGAAGGA	TTATTAACCTG	TTAGAAGACC
9301	AGAGAAATTT	CTTATTGATA	CATCCGAAT	TGGTTTGAT	ATTAGATAAA	CAAAACTATA
9361	ATGGTATCT	AATTACTCCT	GATTAAGTAT	TGATGATTTG	TGACGTAGTC	GAAAGCCGTA
9421	GGAATTAAG	TGCATGTCT	AAGTTAGATC	CAAAATTACA	ATCTATGTAT	CAGAAAGGTA
9481	ATAACCTGTG	GGAAGTATA	GATTAATTTG	TTCCAATTAT	GGGAGAAAAG	ACAATTTGATG
9541	TGATATGCTT	ATTAGAACCA	CTTGCAATTA	CCTTAATTCA	AACTCATGAT	CCTGTTAAAC
9601	AACTAGAGAG	AGCTTTTAA	AATCATGTGT	TATCCGAGAT	GGAATTAATA	TTTGAATCTA
9661	GAGATTCGAT	TAAGGAATTT	CTGAGTGTAG	ATTACATTTGA	TAAATTTTAA	GATATATTTA
9721	ATAAGTCTAC	AATAGATGAA	ATAGCTGAGA	TTTTCTCTTT	TTTTAGAAC	TTTGGGCATC
9781	CTCCATTAGA	AGCTAGTATT	GCAGCAGAAA	AGGTTAGAAA	ATATATGTAT	ATTGGGAAAC
9841	AATTAATAAT	TGACACTATT	AATTAATGTC	ATGCTATCTT	CTGTACAATA	ATAATTAACG
9901	GATATAGAGA	GAGGCATGCT	GGACAGTGGC	CTCCTGTGAC	ATTACCTGAT	CATGCACACG
9961	AATTCATCAT	AAATGCTTAC	GGTCAAACT	CTGCGATATC	ATATGAAAAT	GCTGTTGATT
10021	ATTACCAAG	CTTTATAGGA	ATAAAATTTCA	ATTAATTCAT	AGAGCCTCAG	TTAGATGAGG
10081	ATTGACCAAT	TTATATGAAA	GATTAAGCAT	TATCTCCAAA	AAAATCAAAAT	TGGGACACAG
10141	TTTATCTGC	ATCTAATTTA	CTGTACCCTTA	CTAACGCATC	CAACGAATCA	CGAAGATTAG
10201	TTGAGTATT	TATAGCAGAT	AGTAATTTTG	ATCCTCATCA	GATATTGGAT	TATGTAGATG
10261	CTGGGACTG	GTTAGATGAT	CCAGAATTTA	ATATTCTTTA	TAGTCTTAAA	GAAAAAGAGA
10321	TCAACACAGGA	AGGTAGACTC	TTTGCAAAAA	TGACATACAA	AATGAGAGCT	ACACAAGTTT
10381	TATCAGAGAC	CCTACTTGCA	AATAACATAG	GAAATTCCTT	TCAGAGAAAT	GGGATGGTGA
10441	AGGAGAGAT	TGAATTAATT	AAGAGATTTA	CAACCATATC	AATATCAGGA	GTTCCACGCT
10501	ATAATGAAGT	GTACATTAAT	TCTAAAGGCC	ATACAGATGA	CCTTAAACC	TACAAATAAA
10561	TAAGTATCT	TAATTTGCT	TCTAATCAGA	AATCAAGAA	ATTGAATTC	AACTCAACGG
10621	AAATCTAGAA	TGATGGATAG	GAGAGTCTGA	GCTGTTTCTT	AACACACAGAT	CTCAAAAAT
10681	ACTGCTTTAA	TTGAGATAT	GAATCAACAG	CTCTATTTGG	AGAACCTTGC	AAACCAATAT

TABLE 11 (cont'd)

11521	GATGTTTGT	AAGAAATATT	GGTATCCAT	CAGTTGCCG	ATTGGCTGAT	ATTAAAGAT
11581	TTATTAAAGC	GAATCTATTA	GACCCGAGTG	TTCTTTATAG	GATTATGAT	CAAGAACCAG
11641	GTGAGTCATC	TTTTTTGGAC	TGGGCTTCAG	ATCCATATTC	ATGCAATTTA	CCACAATCC
11701	AAATATTAAC	CACCATGATA	AAAAATATTA	CAGCAAGGAA	TGTATTACAA	GATTACCAA
11761	ATCCATTATT	ATCTGATTTA	TTCAACAATA	CAATGATAGA	AGAAGATGAA	GAATTAGCTG
11821	AGTCCCTGAT	GGACAGGAAG	GTAATCTCC	CTAGAGTTGC	ACATGATATT	CTAGATTAAT
11881	CTCTCACAGG	AAATTAGAAT	GCCATAGCTG	GAATGTTAGA	TACGACAAAA	TCACTAATTC
11941	GGGTTGGCAT	AAATAGAGGA	GGACTGACAT	ATAGTTTGT	GAGGAAATC	AGTAATTAACG
12001	ATCTAGTACA	ATATGAACAA	CTAGAGTGA	CTTTCCGACT	AATTGTBAAT	GATAAATTC
12061	AGTATGAAGA	TATGTGTTCC	GTAGACCTTG	CCATAGCATT	CGGACAAAG	ATGTGATTC
12121	ATTATCAGG	AGGAAGGATG	ATTAAGTGAC	TTGAAACGCC	TGACCCATTA	GAATTACTAT
12181	CTGGGAGT	AATAACAGGA	TCAGAACATT	GTAATAATATG	TTATTTCTCA	GATGGACAA
12241	ACCCATATAC	TTGGATGTAT	TTACCCGGTA	ATATCAAAAT	AGGATCAGCA	GAAACAGSTA
12301	TATCGTCATT	AAGAGTTCTT	TATTTGGAT	CAGTCACCTG	TGAAGATCT	GAAAGCAAT
12361	TAGGATATAT	CAAGAATCTT	AGTAACCTG	CAAAAGCCGC	AAATAGAATA	GCAATGATAT
12421	ATACATGGGC	ATTGGTAAT	GATGAGATAT	CTTGGATGGA	AGCCTCAGAG	ATAGACAAA
12481	CACGTGCAAA	TTTTACACTA	GATAGTCTCA	AAATTTTAAC	ACCGTAGCT	ACATCAACAA
12541	ATTATCACA	CAGATTPAAG	GATACTGCAA	CTCAGATGAA	ATTCTCCAGT	ACATCAATGA
12601	TCAGAGTCAG	CAGATTCAATA	ACAATGTCCA	ATGATPAACAT	GTCTATCAAA	GAAGCTAATG
12661	AAACCAAGA	TACTAATCTT	ATTATCAAC	AAATAATGTT	AACAGATTA	AGTGTTTTCG
12721	AATATTTATT	TAGATTPAAA	GAACACACAG	GACACAACCC	TATAGTTATG	CATCTCCACA
12781	TAGAAGATGA	GTGTTGTATT	AAAGAAAGTT	TTAATGATGA	ACATATTAAT	CCAGAGCTTA
12841	CATTAGATAT	AATTCGATAT	CCTGAAGTA	ATGAATTTAT	TTATGATCAA	ATTGACTCA
12901	AAGATGTGA	CTTATCAAAA	CTTATGTTTA	TTAAGACCA	TTCTTACACA	ATTGATATGA
12961	ATTATGGGA	TGATACTGAC	ATCATACATG	CAATTTCAAT	ATGTACTGCA	ATTACAAATG
13021	CAGATACTAT	GTCAACATTA	GATCGAGATA	ATTTAAAGA	GATPATAGTT	ATTGCAATG
13081	ATGATGATAT	TAATAGCTTA	ATCACTGAAT	TTTGAACCTT	TGACATACTT	GTATTTCTCA
13141	AGACATTTGG	TGGATTATTA	GTAATATCAAT	TTGCAATCAC	TCTTATATAGT	CTAAAAATAG
13201	AAGTAGGGA	TCTCATTTGG	GATTATATTA	TGAGAACACT	GAGACAGACT	TCCCATTTCA
13261	TATTAAAGT	ATTATCTAAT	GCATTATCTC	ATCCTAAAGT	ATTCAAGAGG	TTCTGGGATT
13321	GTGGAGTTT	AAACCCTATT	TATGTCCTTA	ATACTGCTAG	TCAAGACCAG	ATTAACCTTG
13381	CCCTATCTAT	ATGTGAATAT	TCACTAGATC	TATTTATGAG	AGAATGTTG	AATGGTGTAT
13441	CACTTGAAT	ATACATTTGT	GACAGCGATA	TGGAAGTTGC	AAATGATAGG	AAACAACCTT
13501	TTATTTCTAG	ACACCTTTCA	TTTGTTTGTT	GTTTACGAGA	AAATTCATCT	TTCCGACCTA
13561	ACCTGTTAAA	CTTAACATAC	TTGAGAGAGC	TTGATCTATT	GAAACATATAT	CTTGAATTTAA
13621	ATATTAAAGA	AGACCCTACT	CTTAATATATG	TACAATATATC	TGGATTATTA	ATTGAATTCGT
13681	TCCCATCAAC	TGTAACATAC	GTAAGAAGA	CTGCCATCAA	ATATCTAAGG	ATTCCGGGTA
13741	TTAGTCCACC	TGAGGTAAT	GATGATTGGG	ATCCGCTAGA	AGATGAANAAT	ATGCTGCATA
13801	ACATGTCTAA	AACTATAAAT	GATPACTGTA	ATTAAGATPA	TAAAGGGAAT	AAAATTTAACA
13861	ATTCTGGGG	ACTAGCACTT	AAGAATATC	AACTCCTTAA	AATCAGATCT	ATPACAAGTG
13921	ATTCTGATGA	TAATGATAGA	CTAGATGCTA	ATACAAGTGG	TTTGACACTT	CCTCAAGGAG
13981	GGAATTATCT	ATCGATCAAA	TTGAGATTAT	TCGGAATCAA	CAGCACTAGT	TGCTGGAAG
14041	CTCTGAGTT	ATCACAATAT	TTAATGAAGG	AAATCAATPA	AGACAAGGAG	AGGCTCTTCC
14101	TGGAGAGAGG	AGCAGAGCT	ATGCTAGACT	GTTATGATGC	CACATTAAGAT	CCTGAGCTTA
14161	ATTATATATA	TTCAGGTTTG	AATATPAACAG	ATGTAATTTG	TCAACGAGAA	TTGAAATATAT
14221	TTCCTTCAGA	GGTATCATTA	GTAAGTAAAA	AATTAGGAAA	TGTGACACAG	ATTCTTTAACA
14281	GGTAAAGT	ACTGTCAAT	GGGAATCCTA	ATTCAACATG	GATAGGAAT	ATGCAATGTG
14341	AGAGCTTAAT	ATGAGTGAA	TTAATGATA	AGTCATTTGG	ATTAGTACAT	TGTGATATG
14401	AAGAGCTTAT	CGGTAATAT	GAAGAACTG	TTCTACATGA	ACAATTATAGT	GTTATTAAGAA
14461	TTACATACTT	GATTGGGAT	GATGATGTTG	TTTTAGTTTC	CAAAATTATA	CCTAATATCA
14521	GTGGAATG	GTOTAGAATA	CTTATATAT	ATTAATTATA	TTGGAAGAGT	GTAAGTATATA
14581	TATCACTCAA	AACTTCTAAT	CCTGATCAAA	CAGAAATATA	TCTAATTTTCG	AAAGATGCAT

TABLE 11 (cont'd)

15421 TTTAAGCCCTA GGAATAGACA AAAAGTAAGA AAAACATGTA ATATATATAT ACCAACAAGA
15481 GTTCTTCTCT TGTTTGGT

Sub. 9.15
In a third strategy (Figure 8), chimeric PIV3-PIV2 F and HN genes were constructed in which regions of the PIV2 F and HN ORFs encoding the ectodomains and the transmembrane domains were amplified from pLit.PIV32Fhc and pLit.PIV32HNhc, respectively, using PCR, Vent DNA polymerase, and primer pairs specific to PIV2 F (13, 14 in Table 9) and PIV2 HN (15, 16 in Table 9). In parallel, the partial ORFs of PIV3 F and HN genes encoding the ectodomains plus transmembrane domains were deleted from their cDNA subclones pLit.PIV3.F3a and pLit.PIV3.HN4 (Tao et al., J. Virol. 72:2955-2961, 1998, incorporated herein by reference), respectively, using PCR, Vent DNA polymerase, and primer pairs specific to PIV3 F (17, 18 in Table 1) and PIV3 HN (19, 20 in Table 9). The F and HN cDNA fragments of PIV2 and PIV3 were gel purified and ligated to generate pLit.PIV32FCT and pLit.PIV32HNCT, respectively. The chimeric F and HN constructs were digested with PpuMI plus SpeI and assembled together to generate pLit.PIV32CT, which was sequenced across the PIV specific region in its entirety and found to be as designed. The 4 kb BspEI-SpeI fragment from pLit.PIV32CT was introduced into the BspEI-SpeI window of p38'ΔPIV31hc to generate p38'ΔPIV32CT. The 6.5 kb BspEI-SphI fragment from p38'ΔPIV32CT, containing the PIV3-PIV2 F and HN chimeric genes, was introduced into the BspEI-SphI window of pFLC.2G+.hc and pFLCcp45, to generate pFLC.PIV32CT (Table 12, SEQ ID NO:) and pFLC.PIV32CTcp45, respectively. The nucleotide sequence of this *BspEI-SpeI* fragment is submitted in the GenBank (Accession No. pending).

Sub a16

TABLE 12 (SEQUENCE) NO:

Sequence of pFLC.PIV32CT, 15474 bp in sense orientation
(only the insert is shown)

```

1  ACCAAACAAG AGAAGAAACT TGTCTGGGAA TATAAATTTA ACTTTAAATT AACTTAGGAT
61  TAAAGACATT GACTAGAAGG TCAAGAAAAG GGAACCTCTAT AATTTCAAAA ATGTTGAGCC
121 TATTTGATAC ATTTAATGCA CGTAGGCAAG AAAACATAAC AAAATCAGCC GGTGGAGCTA
181 TCATTCTCTG ACAGAAAAAT ACTGTCTCTA TATTCGCCCT TGGACCGACA ATAAGTGATG
241 ATAATGAGAA AATGACATTA GCTCTTCTAT TTCTATCTCA TTCACTAGAT AATGAGAAAC
301 AACATGCACA AAGGGCAGGG TTCTTGGTGT CTTTATTGTC AATGGCTTAT GCCAATCCAG
361 AGCTCTACCT AACACAAAT GGAAGTAATG CAGATGTCAA GTATGTCATA TACATGATTG
421 AGAAAGATCT AAAACGGCAA AAGTATGGAG GATTTGTGGT TAAGACGAGA GAGATGATAT
481 ATGAAAAGAC AACTGATTGG ATATTTGGAA GTGACCTGGA TTATGATCAG GAAACTATGT
541 TGCAGAACGG CAGGAACAAT TCAACAATTG AAGACCTTGT CCACACATTT GGGTATCCAT
601 CATGTTTAG AGCTCTTATA ATACAGATCT GGATAGTTCT GGTCAAAGCT ATCACTAGTA
661 TCTCAGGGTT AAGAAAAGGC TTTTTCACCC GATTGGAAGC TTTCAGACAA GATGGAACAG
721 TGCAGGCAGG GCTGGTATTG AGCGGTGACA CAGTGGATCA GATTGGGTCA ATCATGCGGT
781 CTCAACAGAG CTTGGTAACT CTTATGGTTG AAACATTAAT AACAAATGAAT ACCAGCAGAA
841 ATGACCTCAC AACCATAGAA AAGAATATAC AAATTGTTGG CAACTACATA AGAGATGCAG
901 GTCTCGCTTC ATTCTTCAAT ACAATCAGAT ATGGAATTGA GACCAGAATG GCAGCTTTGA
961 CTCTATCCAC TCTCAGACCA GATATCAATA GATTAAAAGC TTTGATGGAA CTGTATTTAT
1021 CAAAGGGACC ACGCGCTCCT TTCATCTGTA TCCTCAGAGA TCCTATACAT GGTGAGTTCT
1081 CACCAGGCAA CTATCCTGCC ATATGGAGCT ATGCAATGGG GGTGGCAGTT GTACAAAATA
1141 GAGCCATGCA ACAGTATGTG ACGGGAAGAT CATATCTAGA CATTGATATG TTCCAGCTAG
1201 GACAAGCAGT AGCAGCTGAT GCCGAAGCTC AAATGAGCTC AACACTGGAA GATGAACTTG
1261 GAGTGACACA CGAATCTAAA GAAAGCTTGA AGAGACATAT AAGGAACATA AACAGTTCAG
1321 AGACATCTTT CCACAAACCG ACAGGTGGAT CAGCCATAGA GATGGCAATA GATGAAGAGC
1381 CAGAACAATT CGAACATAGA GCAGATCAAG AACAAAATGG AGAACCTCAA TCATCCATAA
1441 TTCAATATGC CTGGGCAGAA GGAAATAGAA GCGATGATCA GACTGAGCAA GACTACAGAA
1501 CTGACAATAT CAAGACCGAA CAACAAAACA TCAGAGACAG ACTAAACAAG AGACTCAACG
1561 ACAAGAAGAA ACAAAGCAGT CAACCACCCA CTAATCCAC AAACAGAACA AACCAGGACG
1621 AAATAGATGA TCTGTTTAAAC GCATTTGGAA GCAACTAATC GAATCAACAT TTTAATCTAA
1681 ATCAATAATA AATAAGAAAA ACTTAGGATT AAAGAATCCT ATCATACCGG AATATAGGGT
1741 GGTAAATTTA GAGTCTGCTT GAAACTCAAT CAATAGAGAG TTGATGGAAA CCGATGCTAA
1801 AAATATCAA ATCATGGATT CTGGGAAGA GGAATCAAGA GATAAATCAA CTAATATCTC
1861 CTCGGCCCTC AACATCATTG AATTCATACT CAGCACCGAC CCCCAGGAAG ACTTATCGGA
1921 AAACGACACA ATCAACACAA GAACCCAGCA ACTCAGTGCC ACCATCTGTC AACCAGAAAT
1981 CAAACCAACA GAAACAAGTG AGAAAGATAG TGGATCAACT GACAAAATA GACTCCGG
2041 GTCATCACAC GAATGTACAA CAGAAGCAAA AGATAGAAAT ATTGATCAGG AAAGTGTACA
2101 GAGAGGACCT GGGAGAAGAA GCAGCTCAGA TAGTAGAGCT GAGACTGTGG TCTCTGGAGG
2161 AATCCCCAGA AGCATCACAG ATTCTAAAAA TGGAAACCAA AACACGGAGG ATATTGATCT
2221 CAATGAAATT AGAAAGATGG ATAAGGACTC TATTGAGGGG AAAATGCGAC AATCTGCAA
2281 TGTTCCAAGC GAGATATCAG GAAGTATGTA CATATTTACA CATATTTACA GTAGAAACAG
2341 TGATCATGGA AGAAGCCTGG AATCTATCAG TACACCTGAT ACAAGATCAA TAAGTGTGTG
2401 TACTGCTGCA ACACCAGATG ATGAAGAAGA AATACTAATG AAAAATAGTA GGACAAAGAA
2461 AAGTTCTTCA ACACATCAAG AAGATGACAA AAGAATTAAA AAAGGGGGAA AAGGGAAAGA
2521 CTGGTTTAAG AAATCAAAAG ATACCGACAA CCAGATACCA ACATCAGACT ACAGATCCAG
2581 ATCAAAAGGG CAGAAGAAAA TCTCAAAGAC AACCAACCAC AACACCGACA CAAAGGGGCA
2641 AACAGAAATA CAGACAGAAAT CATCAGAAAC ACAATCCTCA TCATGGAATC TCATCATCGA
2701 CAACAACACC GACCGGAACG AACAGACAAG CACAACCTCT CCAACAACAA CTTCCAGATC
2761 AACTTATACA AAAGAATCGA TCCGAACAAA CTCTGAATCC AAACCCAAGA CACAAAAGAC
2821 AAATGGAAAG GAAAGGAAGG ATACAGAAGA GAGCAATCGA TTTACAGAGA GGGCAATTAC
2881 TCTATTGCAG AATCTTGGTG TAATTCAATC CACATCAAAA CTAGATTTAT ATCAAGACAA
2941 ACGAGTTGTA TGTGTAGCAA ATGTACTAAA CAATGTAGAT ACTGCATCAA AGATAGATTT
3001 CCTGGCAGGA TTAGTCATAG GGGTTTCAAT GGACAACGAC ACAAATTAAT CACAGATACA
3061 AAATGAAATG CTAACCTCA AAGCAGATCT AAAGAAAATG GACGAATCAC ATAGAAGATT
3121 GATAGAAAAT CAAAGAGAAC AACTGTCATT GATCAGTCA CTAATTTCAA ATCTCAAAAT
3181 TATGACTGAG AGAGGAGGAA AGAAAGACCA AAATGAATCC AATGAGAGAG TATCCATGAT
3241 CAAAACAAAA TTGAAAGAAG AAAAGATCAA GAAGACCAGG TTTGACCCAC TTATGGAGGC
3301 ACAAGGCATT GACAAGAATA TACCCGATCT ATATCGACAT GCAGGAGATA CACTAGAGAA
3361 CGATGTACAA GTTAAATCAG AGATATTAA TTCATACAAT GAGTCAAATG GAACAAGACT
3421 AATACCCAAA AAAGTGAGCA GTACAATGAG ATCACTAGTT GCAGTCATCA ACAACAGCAA
3481 TCTCTACAAA AGCACAAAAC AATCATACAT AAACGAACTC AAACGTTGCA AAAATGATGA
3541 AGAAGTATCT GAATTAATGG ACATGTTCAA TGAAGATGTC AACAAATGCC AATGATCCAA
3601 CAAAGAAACG ACACCGAACA AACAGACAAG AAACAACAGT AGATCAAAAC CTGTCAACAC
3661 ACACAAAATC AAGCAGAATG AAACAACAGA TATCAATCAA TATACAAATA AGAAAACTT

```

TABLE 12 (cont'd)

3721	AGGATTAAAG	AATAAAATTA	TCCTTGTC	AAATGAGTAT	AACTAACTCT	GCAATATACA
3781	CATTCCCAGA	ATCATCATT	TCTGAAAATG	GTCATATAGA	ACCATTACCA	CTCAAAGTCA
3841	ATGAACAGAG	GAAAGCAGTA	CCCCACATTA	GAGTTGCCAA	GATCGGAAAT	CCACCAAAC
3901	ACGGATCCCG	GTATTTAGAT	GTCTTCTTAC	TCCGGCTTCT	CGAGATGGAA	CGAATCAAAG
3961	ACAAATACGG	GAGTGTGAAT	GATCTCGACA	GTGACCCGAG	TTACAAAGTT	TGTGGCTCTG
4021	GATCATTACC	AATCGGATTG	GCTAAGTACA	CTGGGAATGA	CCAGGAATTG	TTACAAGCCG
4081	CAACCAAAC	GGATATAGAA	GTGAGAAGAA	CAGTCAAAGC	GAAAGAGATG	GTTGTTTACA
4141	CGGTACAAAA	TATAAAACCA	GAACGTGTACC	CATGGTCCAA	TAGACTAAGA	AAAGGAATGC
4201	TGTTTCGATG	CAACAAAGTT	GCTCTTGCTC	CTCAATGTCT	TCCACTAGAT	AGGAGCATAA
4261	AATTTAGAGT	AATCTTCGTG	AATTGTACGG	CAATTGGATC	AATAACCTTG	TTCAAAATTC
4321	CTAAGTCAAT	GGCATCACTA	TCTCTACCCA	ACACAATATC	AATCAATCTG	CAGGTACACA
4381	TAAAAACAGG	GGTTCAGACT	GATTCTAAAG	GGATAGTTCA	AATTTTGGAT	GAGAAAGGCG
4441	AAAAATCACT	GAATTTTCATG	GTCCATCTCG	GATTGATCAA	AAGAAAAGTA	GGCAGAAATGT
4501	ACTCTGTTGA	ATACTGTAAA	CAGAAAATCG	AGAAAATGAG	ATTGATATTT	TCTTTAGGAC
4561	TAGTTGGAGG	AATCAGTCTT	CATGTCAATG	CAACTGGGTC	CATATCAAAA	ACACTAGCAA
4621	GTCAGCTGGT	ATTCAAAAGA	GAGATTTGTT	ATCCTTTAAT	GGATCTAAAT	CCGCATCTCA
4681	ATCTAGTTAT	CTGGGCTTCA	TCAGTAGAGA	TTACAAGAGT	GGATGCAATT	TTCCAACCTT
4741	CTTTACCTGG	CGAGTTCAGA	TACTATCCTA	ATATTATTGC	AAAAGGAGTT	GGGAAAATCA
4801	AACAATGGAA	CTAGTAATCT	CTATTTTAGT	CCGGACGAT	CTATTAAGCC	GAAGCAATA
4861	AAGGATAATC	AAAAACTTAG	GACAAAAGAG	GTCAATACCA	ACAACTATTA	GCACTCACAC
4921	TCGCAAGAAT	AAGAGAGAAG	GGACCAAAAA	AGTCAAATAG	GAGAAATCAA	AACAAAAGGT
4981	ACAGAACACC	AGAACAACAA	AATCAAAACA	TCCAACCTAC	TCAAAACAAA	AATTCCAAAA
5041	GAGACCGGCA	ACACAACAAG	CACCTGAACAT	GCATCACCTG	CATCCAATGA	TAGTATGCAT
5101	TTTTGTATTG	TACACTGGAA	TTGTGAGTTC	AGATGCCATT	GCTGGAGATC	GCTGGAGATC
5161	TGTAGGGGTC	ATTCAATCAA	AGATAAGATC	ACTCATGTAC	TACACTGATG	GTGGCGCTAG
5221	CTTTATTGTT	GTAAAATTAC	TACCCAATCT	TCCCCCAAGC	AATGGAACAT	GCAACATCAC
5281	CAGTCTAGAT	GCATATAATG	TTACCCATT	TAAGTTGCTA	ACACCCCTGA	TTGAGAACCCT
5341	GAGCAAAAT	TCTGCTGTTA	CAGATACCAA	ACCCGCGCGA	GAACGATTG	CAGGAGTCGT
5401	TATTGGGCTT	GCTGCACTAG	GAGTAGCTAC	AGCTGCACAA	ATAACCGCAG	CTGTAGCAAT
5461	AGTAAAAGCC	AATGCAAATG	CTGCTGCGAT	AAACAATCTT	GCATCTTCAA	TTCAATCCAC
5521	CAACAAGGCA	GTATCCGATG	TGATAACTGC	ATCAAGAACA	ATTGCAACCG	CAGTTCAAGC
5581	GATTCAAGAT	CACATCAATG	GAGCCATTGT	CAACGGGATA	ACATCTGCAT	CATGCCGTGC
5641	CCATGATGCA	CTAATTGGGT	CAATATTAAA	TTTGTATCTC	ACTGAGCTTA	CTACAATATT
5701	TCATAATCAA	ATAACAAACC	CTGCGCTGAC	ACCACTTTCC	ATCCAAGCTT	TAAGAATCCT
5761	CCTCGGTAGC	ACCTTGCCAA	TTGTCAATTG	ATCCAACTC	AACACAAAAC	TCAACACAGC
5821	AGAGCTGCTC	AGTAGCGGAC	TGTTAACTGG	TCAAATAATT	TCCATTTCCC	CAATGTACAT
5881	GCAAATGCTA	ATTCAAATCA	ATGTTCCGAC	ATTTATAATG	CAACCCGGTG	CGAAGGTAAT
5941	TGATCTAATT	GCTATCTCTG	CAAAACATAA	ATTACAAGAA	GTAGTTGTAC	AAGTTCTCTA
6001	TAGAATTCTA	GAATATGCAA	ATGAACTACA	AAACTACCCA	GCCAATGATT	GTTTCGTGAC
6061	ACCAAATCT	GTATTTTGTA	GATACAATGA	GGGTTCCCCG	ATCCCTGAAT	CACAATATCA
6121	ATGCTTAAGG	GGGAATCTTA	ATTCTTGAC	TTTTACCCCT	ATTATCGGGA	ACTTTCTCAA
6181	GCGATTTCGA	TTTGCCAATG	TGTGCTCTA	TGCCAACTGC	AAATCTTTG	TATGTAAGTG
6241	TGCCGACCCT	CCCCATGTTG	TGTCTCAAGA	TGACAACCAA	GGCATCAGCA	TAATTGATAT
6301	TAAGAGGTGC	TCTGAGATGA	TGCTTGACAC	TTTTTCATTT	AGGATCACAT	CTACATTCAA
6361	TGCTACATAC	GTGACAGACT	TCTCAATGAT	TAATGCAAA	ATTGTACATC	TAAGTCTCT
6421	AGACTTGCTA	AATCAAATCA	ATTCAATAAA	CAAATCTCTT	AAAAGTGCTG	AGGATTGGAT
6481	TGCAGATAGC	AACCTCTTCG	CTAATCAAGC	CAGAACAGCC	AAGACACTTT	ATTCATAAG
6541	TGCAATCGCA	TTAATACTAT	CAGTGATTAC	TTTGGTTGTT	GTGGGATTGC	TGATTGCCTA
6601	CATCATCAAG	TATTACAGAA	TTCAAAAGAG	AAATCGAGTG	GATCAAAATG	ACAAGCCATA
6661	TGTACTAACA	AACAATAAAC	ATATCTACAG	ATCATTAGAT	ATTAAAATTA	TAAAAAACTT
6721	AGGAGTAAAG	TTACGCAATC	CAACTCTACT	CATATAATTG	AGGAAGGACC	CAATAGACAA
6781	ATCCAAATTC	GAGATGGAAT	ACTGGAAGCA	TACCAATCAC	GGAAAGGATG	CTGGTAATGA
6841	GCTGGAGACG	TCTATGGCTA	CTCATGGCAA	CAAGCTCACT	AATAAGACTG	CCACAATTCT
6901	TGGCATATGC	ACATTAATTG	TGCTATGTTT	AAGTATTCTT	CATGAGATAA	TTCATCTTGA
6961	TGTTTCTCT	GGTCTTATGA	ATTCTGATG	GTCACAGCAA	GGCATTATTC	AGCCTATCAT
7021	AGAATCATT	AAATCATTGA	TTGCTTTGGC	CAACCAGATT	CTATATAATG	TTGCAATAGT
7081	AATTCCTCTT	AAAATTGACA	GTATCGAAAC	TGTAATACTC	TCTGCTTTAA	AAGATATGCA
7141	CACCGGGAGT	ATGTCCAATG	CCAACCTGCAC	GCCAGGAAAT	CTGCTTCTGC	ATGATGCAGC
7201	ATACATCAAT	GGAAATAACA	AATTCCTTGT	ACTTGAATCA	TACAATGGGA	CGCTAAATA
7261	TGGACCTCTC	CTAAATATAC	CCAGCTTTAT	CCCCCAGCA	ACATCTCCCC	ATGGGTGTAC
7321	TAGAATACCA	TCATTTTCAC	TCATCAAGAC	CCATTGGTGT	TACACTCACA	ATGTAATGCT
7381	TGGAGATTGT	CTTGATTTC	CGGCATCTAA	CCAGTATTTA	TCAATGGGGA	TAATACAACA
7441	ATCTGCTGCA	GGGTTTCCAA	TTTTTCAGGAC	TATGAAAACC	ATTTACCTAA	GTGATGGAAT
7501	CAATCGCAAA	AGCTGTTTCA	TCACTGCTAT	ACCAGGAGGT	TGTGCTTGT	ATTGCTATGT
7561	AGCTACAAGG	TCTGAAAAAG	AAGATTATGC	CACGACTGAT	CTAGCTGAAC	TGAGACTTGC

TABLE 12 (cont'd)

7621	TTTCTATTAT	TATAATGATA	CCTTTATTGA	AAGAGTCATA	TCTCTTCCAA	ATACAACAGG
7681	GCAGTGGGCC	ACAATCAACC	CTGCAGTCGG	AAGCGGGATC	TATCATCTAG	GCTTTATCTT
7741	ATTTCTGTGA	TATGGTGGTC	TCATAAATGG	GACTACTTCT	TACAATGAGC	AGTCCTCAGC
7801	CTATTTTATC	CCAAAACATC	CCAACATAAC	TTGTGCCGGT	AACCTCCAGCA	AACAGGCTGC
7861	AATAGCACGG	AGTTCCTATG	TCATCCGTTA	TCACCTCAAC	AGGTTAATTC	AGAGTGCTGT
7921	TCTTATTTGT	CCATTGTCTG	ACATGCATAC	AGAAGAGTGT	AATCTAGTTA	TGTTTAAACAA
7981	TTCCCAAGTC	ATGATGGGTG	CAGAAGGTAG	GCTCTATGTT	ATTGGTAATA	ATTTGTATTA
8041	TTATCAACGC	AGTTCCTCTT	GGTGGTCTGC	ATCGCTCTTT	TACAGGATCA	ATACAGATTT
8101	TTCTAAAGGA	ATTCCTCCGA	TCATTGAGGC	TCAATGGGTA	CCGTCTATC	AAGTTCCTCG
8161	TCCTGGAGTC	ATGCCATGCA	ATGCAACAAG	TTTTTGCCCT	GCTAATTGCA	TCACAGGGGT
8221	GTACGCAGAT	GTGTGGCCGC	TTAATGATCC	AGAACTCATG	TCACGTAATG	CTCTGAACCC
8281	CAACTATCGA	TTTGCTGGAG	CCTTTCTCAA	AAATGAGTCC	AACCGAACTA	ATCCCACATT
8341	CTACACTGCA	TCGGCTAACT	CCCTCTTAAA	TACTACCGGA	TTCAACAACA	CCAATCACAA
8401	AGCAGCATAT	ACATCTTCAA	CCTGCTTTAA	AAACACTGGA	ACCCAAAAAA	TTTATTGTTT
8461	AATAATAATT	GAAATGGGCT	CATCTCTTTT	AGGGGAGTTC	CAAATAATAC	CATTTTAAAG
8521	GGAACATAATG	CTTTAATCAT	AATTAACCAT	AATATGCATC	AATCTATCTA	TAATACAAGT
8581	ATATGATAAG	TAATCAGCAA	TCAGACAATA	GACAAAAGGG	AAATATAAAA	AACTTAGGAG
8641	CAAAGCGTGC	TCGGGAAATG	GACACTGAAT	CTAACAATGG	CACTGTATCT	GACATACTCT
8701	ATCCTGAGTG	TCACCTTAAC	TCTCCTATCG	TTAAAGGTAA	AATAGCACAA	TTACACACTA
8761	TTATGTAGTCT	ACCTCAGCCT	TATGATATGG	ATGACGACTC	AATACTAGTT	ATCAGTAGAC
8821	AGAAAAATAAA	ACTTAATAAA	TTGGATAAAA	GACAACGATC	TATTAGAAGA	TTAAAAATTAA
8881	TATTAACCTGA	AAAAGTGAAT	GACTTAGGAA	AATACACATT	TATCAGATAT	CCAGAAATGT
8941	CAAAAGAAAT	GTTCAAATTA	TATATACCTG	GTATTAACAG	TAAAGTGACT	GAATTATTAC
9001	TTAAAGCAGA	TAGAACATAT	AGTCAAAATG	CTGATGGATT	AGAGAGATCTA	TGGATTAATG
9061	TGCTATCAAAA	ATTAGCCTCA	AAAAATGATG	GAAACAAATTA	TGATCTTAAT	GAAGAAATTA
9121	ATAATATATC	GAAAGTTCAC	ACAACCTATA	AATCAGATAA	ATGGTATAAT	CCATTCAAAA
9181	CATGGTTTAC	TATCAAGTAT	GATATGAGAA	GATTACAAAA	AGCTCGAAAT	GAGATCACTT
9241	TTAATGTTGG	GAAGGATTAT	AACCTGTTAG	AAGACCAGAA	GAATTTCTTA	TTGATACATC
9301	CAGAATTGGT	TTTGATATTA	GATAAACAAA	ACTATAATGG	TTATCTAATT	ACTCCTGAAT
9361	TAGTATTGAT	GTATTGTGAC	GTAGTCGAAG	GCCGATGGAA	TATAAGTGCA	TGTGCTAAGT
9421	TAGATCCAAA	ATTACAATCT	ATGTATCAGA	AAGGTAATAA	CCTGTGGGAA	GTGATAGATA
9481	AATTGTTTCC	AATTATGGGA	GAAAAGACAT	TTGATGTGAT	ATCGTTATTA	GAACCACTTG
9541	CATTATCCTT	AATTCAAATC	CATGATCCTG	TTAAACAAC	AAGAGGAGCT	TTTTTAAATC
9601	ATGTGTTATC	CGAGATGGAA	TTAATATTTG	AATCTAGAGA	ATCGATTAA	GAATTTCTGA
9661	GTGTAGATTA	CATTGATAAA	ATTTTAGATA	TATTTAATAA	GTCTACAATA	GATGAAATAG
9721	CAGAGATTTT	CTCTTTTTTT	AGAACATTTG	GGCATCCTCC	ATTAGAAGCT	AGTATTGCAG
9781	CAGAAAAGGT	TAGAAAATAT	ATGTATATTT	GAAAAACAAT	AAAATTTGAC	ACTATTAATA
9841	AATGTCATGC	TATCTTCTGT	ACAATAATAA	TTAACGGATA	TAGAGAGAGG	CATGTTGGAC
9901	AGTGGCCTCC	TGTGACATTA	CCTGATCATG	CACACGAATT	CATCATAAAT	GCTTACGGTT
9961	CAAACTCTGC	GATATCATAT	GAAAATGCTG	TTGATTATTA	CCAGAGCTTT	ATAGGAATAA
10021	AATTCAATAA	ATTCATAGAG	CCTCAGTTAG	ATGAGGATTT	GACAATTTAT	ATGAAAGATA
10081	AAGCATTATC	TCCAAAAAAA	TCAAAATGGG	ACACAGTTTA	TCTTGCATCT	AATTTACTGT
10141	ACCGTACTAA	CGCATCCAAC	GAATCAGGAA	GATTAGTTGA	AGTATTTATA	CGAGATAGTA
10201	AATTTGATCC	TCATCAGATA	TTGGATTATG	TAGAATCTGG	GGACTGGTTA	GATGATCCAG
10261	AATTTAATAT	TTCTTATAGT	CTTAAAGAAA	AAGAGATCAA	ACAGGAAGGT	AGACTCTTTG
10321	CAAAAATGAC	ATACAAAATG	AGAGCTACAC	AAGTTTTATC	AGAGACACTA	CTTGCAAAAT
10381	ACATAGGAAA	ATTCTTTCAA	GAAAATGGGA	TGGTGAAGGG	AGAGATTGAA	TTACTTAAGA
10441	GATTAACAAC	CATATCAATA	TCAGGAGTTC	CACGGTATAA	TGAAGTGATC	AATAATTCTA
10501	AAAGCCATAC	AGATGACCTT	AAAACCTACA	ATAAAATAAG	TAATCTTAAT	TTGTCTTCTA
10561	ATCAGAAATC	AAAGAAATTT	GAATTCAAGT	CAACGGATAT	CTACAATGAT	GGATACGAGA
10621	CTGTGAGCTG	TTTCTTAACA	ACAGATCTCA	AAAAATACTG	TCTTAATTGG	AGATATGAAT
10681	CAACAGCTCT	ATTTGGAGAA	ACTTGCAACC	AAATATTTGG	ATTAAATAAA	TTGTTTAATT
10741	GGTTACACCC	TCGTCTTGAA	GGAAGTACAA	TCTATGTAGG	TGATCCTTAC	TGTCCTCCAT
10801	CAGATAAAGA	ACATATATCA	TTAGAGGATC	ACCCTGATTG	TGGTTTTTAC	GTTTCATAACC
10861	CAAGAGGGGG	TATAGAAGGA	TTTTGTCTAA	AATTATGGAC	ACTCATATCT	ATAAGTGCAA
10921	TACATCTAGC	AGCTGTTAGA	ATAGGCGTGA	GGGTGACTGC	AATGGTTCAA	GGAGACAATC
10981	AAGCTATAGC	TGTAACCACA	AGAGTACCCA	ACAATTATGA	CTACAGAGTT	AAGAAGGAGA
11041	TAGTTTATAA	AGATGTAGTG	AGATTTTTTG	ATTCATTAA	AGAAGTGATG	GATGATCTAG
11101	GTCATGAAT	TAAATTAAAT	GAAACGATTA	TAAGTAGCAA	GATGTTCTA	TATAGCAAAA
11161	GAATCTATTA	TGATGGGAGA	ATTCTTCTCT	AAGCTCTAAA	AGCATTATCT	AGATGTGTCT
11221	TCTGGTCAGA	GACAGTAATA	GACGAAACAA	GATCAGCATC	TTCAAATTTG	GCAACATCAT
11281	TTGCAAAAGC	AATTGAGAAT	GGTTATTAC	CTGTTCTAGG	ATATGCATGC	TCAATTTTAA
11341	AGAATATTCA	ACAACATAT	ATTGCCCTTG	GGATGAATAT	CAATCCAAT	ATAACACAGA
11401	ATATCAGAGA	TCAGTATTTT	AGGAATCCAA	ATTGGATGCA	ATATGCCTCT	TTAATACCTG
11461	CTAGTGTTGG	GGGATTCAAT	TACATGGCCA	TGTCAAGATG	TTTTGTAAGG	AATATTGGTG

TABLE 12 (cont'd)

11521	ATCCATCAGT	TGCCGCATTG	GCTGATATTA	AAAGATTTAT	TAAGGCGAAT	CTATTAGACC
11581	GAAGTGTCT	TTATAGGATT	ATGAATCAAG	AACCAGGTGA	GTCATCTTTT	TTGGACTGGG
11641	CTTCAGATCC	ATATTTCATGC	AATTTTACCAC	AATCTCAAAA	TATAACCACC	ATGATAAAAA
11701	ATATAACAGC	AAGGAATGTA	TTACAAGATT	CACCAAATCC	ATTATTATCT	GGATTATTCA
11761	CAAATACAAT	GATAGAAGAA	GATGAAGAAT	TAGCTGAGTT	CCTGATGGAC	AGGAAGGTAA
11821	TTCTCCCTAG	AGTTGCACAT	GATATTCTAG	ATAATTCTCT	CACAGGAATT	AGAAATGCCA
11881	TAGCTGGAAT	GTTAGATACG	ACAAAATCAC	TAATTCGGGT	TGGCATAAAT	AGAGGAGGAC
11941	TGACATATAG	TTTGTGAGG	AAAATCAGTA	ATTACGATCT	AGTACAATAT	GAAACACTAA
12001	GTAGGACTTT	GCGACTAATT	GTAAGTGATA	AAATCAAGTA	TGAAGATATG	TGTTCCGGTAG
12061	ACCTTGCCAT	AGCATTGCCA	CAAAAGATGT	GGATTCATTT	ATCAGGAGGA	AGGATGATAA
12121	GTGGACTTGA	AACGCCCTGAC	CCATTAGAAT	TACTATCTGG	GGTAGTAATA	ACAGGATCAG
12181	AACATTGTAA	AATATGTTAT	TCTTCAGATG	GCACAAACCC	ATATACTTGG	ATGTATTTAC
12241	CCGGTAATAT	CAAAAATAGGA	TCAGCAGAAA	CAGGTATATC	GTCATTAAGA	GTTCTTTATT
12301	TTGGATCAGT	CACTGATGAA	AGATCTGAAG	CACAATTAGG	ATATATCAAG	AATCTTAGTA
12361	AACCTGCAAA	AGCCGCAATA	AGAATAGCAA	TGATATATAC	ATGGGCATTT	GGTAATGATG
12421	AGATATCTTG	GATGGAAGCC	TCACAGATAG	CACAAACACG	TGCAAATTTT	ACACTAGATA
12481	GTCTCAAAAT	TTTAACACCG	GTAGCTACAT	CAACAAATTT	ATCACACAGA	TTAAAGGATA
12541	CTGCAACTCA	GATGAAATTC	TCCAGTACAT	CATTGATCAG	AGTCAGCAGA	TTCATAACAA
12601	TGTCCAATGA	TAACATGTCT	ATCAAAGAAG	CTAATGAAAC	CAAAGATACT	AATCTTATTT
12661	ATCAACAAAT	AATGTTAACA	GGATTAAAGT	TTTTTCGAATA	TTTATTTAGA	TTAAAGAAAA
12721	CCACAGGACA	CAACCCTATA	GTTATGCATC	TGCACATAGA	AGATGAGTGT	TGTATTAAAG
12781	AAAAGTTTTAA	TGATGAACAT	ATTAATCCAG	AGTCTACATT	AGAATTAATT	CGATATCCTG
12841	AAAGTAATGA	ATTTATTTAT	GATAAAGACC	CACTCAAAGA	TGTGGACTTA	TCAAAACTTA
12901	TGGTTATTAA	AGACCAATCT	TACACAATTT	ATATGAAATTA	TTGGGATGAT	ACTGACATCA
12961	TACATGCAAT	TTCAATATGT	ACTGCAATTA	CAATAGCAGA	TACTATGTCA	CAATTAGATC
13021	GAGATAATTT	AAAAGAGATA	ATAGTTATTG	CAAATGATGA	TGATATTAAT	AGCTTAATCA
13081	CTGAATTTTT	GACTCTTGAC	ATACTTGTAT	TTCTCAAGAC	ATTTGGTGGA	TTATTAGTAA
13141	ATCAATTTTG	ATACACTCTT	TATAGTCTAA	AAATAGAAGG	TAGGGATCTC	ATTTGGGATT
13201	ATATAATGAG	AACACTGAGA	GATACTTCCC	ATTCAATATT	AAAAGTATTA	TCTAATGCAT
13261	TATCTCATCC	TAAAGTATTC	AAGAGGTTCT	GGGATTGTGG	AGTTTTAAAC	CCTATTTATG
13321	GTCTTAATAC	TGCTAGTCAA	GACCAGATAA	AACCTGCCCT	ATCTATATGT	GAATATTCAC
13381	TAGATCTATT	TATGAGAGAA	TGGTTGAATG	GTGTATCACT	TGAAATATAC	ATTTGTGACA
13441	GCGATATGGA	AGTTGCAAAAT	GATGGAATG	AAGCCTTTAT	TTCTAGACAC	CTTTAGTTTG
13501	TTTGTGTTTT	AGCAGAAATT	GCATCTTTCT	GACCTAACCT	GTTAAACTTA	ACATACTTGG
13561	AGAGACTTGA	TCTATTGAAA	CAATATCTTG	AATTAATAT	TAAAGAAGAC	CCTACTCTTA
13621	AATATGTACA	AATATCTGGA	TTATTAAATTA	AATCGTTCCC	ATCAACTGTA	ACATACGTAA
13681	GAAAGACTGC	AATCAAATAT	CTAAGGATTC	GCGGTATTAG	TCCACCTGAG	GTAATTGATG
13741	ATTGGGATCC	GGTAGAAGAT	GAAAAATATG	TGGATAACAT	TGTCAAAACT	ATAAATGATA
13801	ACTGTAATAA	AGATAATAAA	GGGAATAAAA	TTAACAATTT	CTGGGGACTA	GCACTTAAGA
13861	ACTATCAAGT	CCTTAAAATC	AGATCTATAA	CAAGTGATTC	TGATGATAAT	GATAGACTAG
13921	ATGCTAATAC	AAGTGGTTTG	ACACTTCCCT	AAGGAGGGAA	TTATCTATCG	CATCAATTGA
13981	GATTATTTCG	AATCAACAGC	ACTAGTTGTC	TGAAAGCTCT	TGAGTTATCA	CAAATTTTAA
14041	TGAAGGAAGT	CAATAAAGAC	AAGGACAGGC	TCTTCCTGGG	AGAAGGAGCA	GGAGCTTAGC
14101	TAGCATGTTA	TGATGCCACA	TTAGGACCTG	CAGTTAATTA	TTATAATTCA	GGTTTGAATA
14161	TAACAGATGT	AATTGGTCAA	CGAGAATTGA	AAATATTTCC	TTCAGAGGTA	TCATTAGTAG
14221	GTAAAAAATT	AGGAAATGTG	ACACAGATTC	TTAACAGGGT	AAAAGTACTG	TTCAATGGGA
14281	ATCCTAATTC	AACATGGATA	GGAAATATGG	AATGTGAGAG	CTTAATATGG	AGTGAATTAA
14341	ATGATAAGTC	CATTGGATTA	GTACATTGTG	ATATGGAAGG	AGCTATCGGT	AAATCAGAAG
14401	AAACTGTTCT	ACATGAACAT	TATAGTGTTA	TAAGAATTAC	ATACTTGATT	GGGGATGATG
14461	ATGTTGTTTT	AGTTTCCAAA	ATTATACCTA	CAATCACTCC	GAATTGGTCT	AGAATACTTT
14521	ATCTATATAA	ATTATATTGG	AAAGATGTAA	GTATAATATC	ACTCAAAACT	TCTAATCTTG
14581	CATCAACAGA	ATTATATCTA	ATTTTCGAAAG	ATGCATATTG	TACTATAATG	GAACCTAGTG
14641	AAATTGTTTT	ATCAAAACTT	AAAAGATTGT	CACTCTTGGA	AGAAAATAAT	CTATTAAAT
14701	GGATCATTTT	ATCAAAAGAAG	AGGAATAATG	AATGGTTACA	TCATGAAATC	AAAGAAGGAG
14761	AAAGAGATTA	TGGAATCATG	AGACCATATC	ATATGGCACT	ACAAATCTTT	GGATTTCAAA
14821	TCAATTTTAA	TCATCTGGCG	AAAGAATTTT	TATCAACCCC	AGATCTGACT	AATATCAACA
14881	ATATAATCCA	AAGTTTTCAG	CGAACAATAA	AGGATGTTTT	ATTTGAATGG	ATTAATATAA
14941	CTCATGATGA	TAAGAGACAT	AAATTAGGCG	GAAGATATAA	CATATTCCCA	CTGAAAAATA
15001	AGGGAAAGTT	AAGACTGCTA	TCGAGAAGAC	TAGTATTAAG	TTGGATTTCA	TTATCATTAT
15061	CGACTCGATT	ACTTACAGGT	CGTTTCTCTG	ATGAAAAATT	TGAACATAGA	GCACAGACTG
15121	GATATGTATC	ATTAGCTGAT	ACTGATTTAG	AATCATTAAT	GTTATTGTCT	AAAAACATCA
15181	TTAAGAATTA	CAGAGAGTGT	ATAGGATCAA	TATCATATTG	GTTTCTAACC	AAAGAAGTTA
15241	AAATACTTAT	GAAATTGATC	GGTGGTGCTA	AATTATTAGG	AATTCCTCAG	CAATATAAAG
15301	ACCCCGAAGA	CCAGTTATTA	GAAAACTACA	ATCAACATGA	TGAATTTGAT	ATCGATTAAA
15361	ACATAAATAC	AATGAAGATA	TATCCTAAC	TTTATCTTTA	AGCCTAGGAA	TAGACAAAAA

TABLE 12 (cont'd)

15421 GTAAGAAAAA CATGTAATAT ATATATACCA AACAGAGTTC TTCTCTTGTT TGGT

[illegible]

The cDNA engineering was designed so that the final PIV3-2 antigenomes conformed to the rule of six (Calain et al., *J. Virol.* 67:4822-30, 1993; Durbin et al., *Virology* 234:74-83, 1997, each incorporated herein by reference). The PIV3-2 insert in pFLC.PIV32TM is 15498 nt in length, and that in pFLC.PIV32CT is 15474 nt in length. These total lengths do not include two 5'-terminal G residues contributed by the T7 promoter, because it is assumed that they are removed during recovery.

Transfection and recovery of recombinant chimeric PIV3-PIV2 viruses

Hep-2 cell monolayers were grown to confluence in six-well plates, and transfections were performed essentially as described (Tao et al., 72:2955-2961, 1998, incorporated herein by reference). The Hep-2 monolayer in one well was transfected with 5 µg PIV3-PIV2 antigenomic cDNA and three support plasmids, 0.2 µg pTM(N), 0.2 µg pTM(PnoC), 0.1 µg pTM(L) in 0.2 ml of MEM containing 12 µl LipofectACE (Life Technologies). The cells were infected simultaneously with MVA-T7 at a multiplicity of infection (MOI) of 3 in 0.8 ml of serum-free MEM containing 50 µg/ml gentamicin and 2 mM glutamine. The chimeric antigenomic cDNA pFLC.2G+.hc (Tao et al., *J. Virol.* 72:2955-2961, 1998), was transfected in parallel as a positive control. After incubation at 32°C for 12 hours, the transfection medium was replaced with 1.5 ml of fresh serum-free MEM supplemented with 50 µg/ml gentamicin and 2 mM glutamine. Transfected cells were incubated at 32°C for two additional days. Gamma-irradiated porcine trypsin (p-trypsin; T1311, Sigma, St Louis, MO) was added to a final concentration of 0.5 µg/ml on day 3 post transfection. Cell culture supernatants were harvested and passaged (referred to as passage 1) onto fresh Vero cell monolayers in T25 flasks. After overnight adsorption, the transfection harvest was replaced with fresh VP-SFM supplemented with 0.5 µg/ml p-trypsin. Cultures from passage 1 were incubated at 32°C for 4 days, and the amplified virus was harvested and further passaged on Vero cells (referred to as passage 2) for another 4 days at 32°C in the presence of 0.5 µg/ml p-trypsin. The presence of viruses in the passage 2 cultures was determined by hemadsorption with 0.2% guinea pig red blood cells (RBCs). Viruses were further purified by three consecutive terminal dilutions performed using Vero cells maintained in VP-SFM supplemented with 2 mM glutamine, 50 µg/ml gentamicin, and 0.5 µg/ml p-trypsin. Following the third terminal dilution, virus was further amplified three times on Vero cells, and this virus suspension was used for further characterization *in vitro* and *in vivo*.

Confirmation of the chimeric nature of vRNA using sequencing and restriction analysis of PCR products

For analysis of the genetic structure of vRNAs, the recombinant PIVs were amplified on LLC-MK2 cells and concentrated. vRNA was extracted from the viral pellets and reverse transcribed using the Superscript Preamplification System. RT-PCR was performed using the Advantage cDNA synthesis kit and primer pairs specific to PIV2 or PIV3 (21, 22 or 23, 24 in Table 9). RT-PCR products were either analyzed by restriction digestion or gel purified and analyzed by sequencing.

Replication of PIVs in LLC-MK2 cells

Growth of the PIV viruses in tissue culture was evaluated by infecting confluent LLC-MK2 cell monolayers on six-well plates in triplicate at an MOI of 0.01. The inoculum was removed after absorption for 1 hour at 32°C. Cells were washed 3 times with serum-free OptiMEM I, fed with 2 ml/well of OptiMEM I supplemented with 50 µg/ml gentamicin and 0.5 µg/ml p-trypsin, and incubated at 32°C. At each 24 hour interval, a 0.5 ml aliquot of medium was removed from each well and flash-frozen, and 0.5 ml fresh medium with p-trypsin was added to the cultures. The virus in the aliquots was titrated at 32°C on LLC-MK2 cell monolayers using fluid overlay as previously described (Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference), and the endpoint of the titration was determined by hemadsorption, and the titers are expressed as log₁₀TCID₅₀/ml.

Replication of recombinant chimeric PIV3-PIV2 viruses at various temperatures

Viruses were serially diluted in 1X L15 supplemented with 2 mM glutamine and 0.5 µg/ml p-trypsin. Diluted viruses were used to infect LLC-MK2 monolayers in 96 well plates. Infected plates were incubated at various temperatures for 7 days as described (Skiadopoulos et al., *Vaccine* 18:503-510, 1999, incorporated herein by reference). Virus titers were determined as above.

Replication, immunogenicity, and protective efficacy of recombinant chimeric PIV3-PIV2 viruses in the respiratory tract of hamsters

Golden Syrian hamsters in groups of six were inoculated intranasally with 10^{5.3} TCID₅₀ of recombinant or biologically-derived viruses. Four days after inoculation, hamsters were sacrificed and their lungs and nasal turbinates were harvested and prepared for

quantitation of virus as described (Skiadopoulos et al., Vaccine 18:503-510, 1999). The titers are expressed as mean \log_{10} TCID₅₀/gram of tissue for each group of six hamsters.

Hamsters in groups of 12 were infected intranasally with $10^{5.3}$ TCID₅₀ of viruses on day 0, and six hamsters from each group were challenged four weeks later with 10^6 TCID₅₀ of PIV1 or 10^6 TCID₅₀ of PIV2. Hamsters were sacrificed 4 days after challenge and their lungs and nasal turbinates were harvested. Challenge virus titers in the harvested tissue was determined as previously described (Tao et al., J. Virol. 72:2955-2961, 1998). The virus titers are expressed as mean \log_{10} TCID₅₀/gram of tissue for each group of six hamsters. Serum samples were collected three days prior to inoculation and on day 28, and hemagglutination-inhibition antibody (HAI) titers against PIV1, PIV2, and PIV3 were determined as previously described (van Wyke Coelingh et al., Virology 143:569-582, 1985). The titers are expressed as reciprocal mean \log_2 .

Replication, immunogenicity, and protective efficacy of recombinant chimeric PIV3-PIV2 viruses in African green monkeys (AGMs)

AGMs in groups of 4 were infected intranasally and intratracheally with 10^5 TCID₅₀ of virus at each site on day 0. Nasal/throat (NT) swab specimens and tracheal lavages were collected for 12 and 5 days, respectively, as previously described (van Wyke Coelingh et al., Virology 143:569-582, 1985). On day 29, immunized AGMs were challenged intranasally and intratracheally with 10^5 TCID₅₀ of PIV2/V94 at each site. NT swab specimens and tracheal lavages were collected for 10 and 5 days, respectively. Pre-immunization, post-immunization, and post challenge serum samples were collected on days -3, 28, and 60, respectively. Virus titers in the NT swab specimens and in tracheal lavages were determined as previously described (Tao et al., J. Virol. 72:2955-2961, 1998). Titers are expressed as \log_{10} TCID₅₀/ml. Serum neutralizing antibody titers against PIV1 and PIV2 were determined as previously described (van Wyke Coelingh et al., Virology 143:569-582, 1985), and the titers are expressed as reciprocal mean \log_2 .

Replication and immunogenicity of recombinant chimeric PIV3-PIV2 viruses in chimpanzees

Chimpanzees in groups of 4 were infected intranasally and intratracheally with 10^5 TCID₅₀ of PIV2/V94 or rPIV3-2TM on day 0 as previously described (Whitehead et al., J. Virol. 72:4467-4471, 1998). NT swab specimens were collected daily for 12 days and tracheal lavages were obtained on days 2, 4, 6, 8, and 10. Virus titers in the specimens were

determined as previously described (Tao et al., *J. Virol.* 72:2955-2961, 1998). The peak virus titers are expressed as mean \log_{10} TCID₅₀/ml. Pre-immunization and post-immunization serum samples were collected on days -3 and 28, respectively. Serum neutralizing antibody titers against PIV1 and PIV2 were determined as previously described (van Wyke Coelingh et al., *Virology* 143:569-582, 1985), and the titers are expressed as reciprocal mean \log_2 .

Viable recombinant chimeric virus was not recovered from PIV3-PIV2 chimeric cDNA encoding the complete PIV2 F and HN proteins

The construction of the PIV3-PIV2 chimeric cDNA, in which the F and HN ORFs of the JS wild type PIV3 were replaced by those of PIV2/V94, is described above and summarized in Figure 6. The final plasmid construct, pFLC.PIV32hc (Figure 6), encodes a PIV3-PIV2 chimeric antigenomic RNA of 15492 nt, which conforms to the rule of six.

HEp-2 cell monolayers were transfected with pFLC.PIV32hc along with the three support plasmids pTM(N), pTM(PnoC), and pTM(L) using LipofectACE, and the cells were simultaneously infected with MVA-T7 as previously described (Tao et al., *J. Virol.* 72:2955-2961, 1998, incorporated herein by reference). Virus was not recovered from several initial transfections using pFLC.PIV32hc, while chimeric viruses were recovered from all the transfections using control plasmid pFLC.2G+.hc.

Consistent with these results is the possibility that a mutation occurred outside of the 4 kb BspEI-SpeI segment of pFLC.PIV32hc that prevented the recovery of rPIV3-2 virus from cells transfected with this cDNA clone. To examine this possibility, the BspEI-SpeI fragments of p38' Δ PIV31hc and p38' Δ PIV32hc were exchanged. The regenerated p38' Δ PIV31hc and p38' Δ PIV32hc were identical to those in Figure 6 except that the SpeI-SphI fragments containing PIV3 L gene sequences were exchanged. The BspEI-SphI fragments of these two regenerated cDNAs were introduced into the BspEI-SphI window of a PIV3 full-length clone, p3/7-(131)2G+, in five separate independent ligations to give 10 pFLC.2G+.hc and pFLC.PIV32hc clones (2 clones selected from each ligation), respectively. (Note that the PIV3 sequences outside of the BspEI-SphI window of p3/7-(131)2G+, pFLC.2G+.hc, and pFLC.PIV32hc are identical). Thus, this would have replaced any PIV3 backbone sequence which might have acquired a spurious mutation with sequence known to be functional. Furthermore, the functionality of the backbone was reevaluated in parallel. None of the 10 pFLC.PIV32hc cDNA clones yielded viable virus, but each of the 10 pFLC.2G+.hc cDNA clones yielded viable virus. Virus was not recovered from pFLC.PIV32hc despite passaging the transfection harvest in a fashion similar to that used

successfully to recover the highly defective PIV3 C-knock out recombinant (Durbin et al., Virology 261:319-30, 1999, incorporated herein by reference). Since each of the unique components used to generate the pFLC.PIV32hc was used to successfully generate other recombinant viruses except the cytoplasmic tail domains of F and HN, it is highly unlikely that errors in the cDNA account for the failure to yield recombinant virus in this case. Rather, the favored interpretation is that the full-length PIV2 F and HN glycoproteins are not compatible with one or more of the PIV3 proteins needed for virus growth.

Recovery of chimeric viruses from PIV3-PIV2 chimeric cDNAs encoding the chimeric PIV3-PIV2 F and HN proteins

Using two other strategies, new chimeric PIV3-PIV2 antigenomic cDNAs were constructed, in which the ectodomain or the ectodomain and the transmembrane domain of PIV3 F and HN glycoproteins were replaced by their PIV2 counterparts. The construction of the four full-length cDNAs, namely pFLC.PIV32TM, pFLC.PIV32TMcp45, pFLC.PIV32CT, and pFLC.PIV32CTcp45, is described above and summarized in Figures 7, 8, and 9. The PIV3-2 inserts in the final plasmids pFLC.PIV32TM and pFLC.PIV32CT, in which the F and HN genes encoded chimeric glycoproteins, were 15498 nt and 15474 nt in length, respectively, and conformed to the rule of six (Calain et al., J Virol. 67:4822-30, 1993; Durbin et al., Virology 234:74-83, 1997, each incorporated herein by reference). The authenticity of those four constructs was confirmed by sequencing of the BspEI-SphI region and by restriction analysis.

Recombinant chimeric viruses were recovered from full-length clones pFLC.PIV32TM, pFLC.PIV32CT, pFLC.PIV32TMcp45, or pFLC.PIV32CTcp45 and were designated rPIV3-2TM, rPIV3-2CT, rPIV3-2TMcp45, and rPIV3-2CTcp45, respectively. These viruses were biologically cloned by 3 consecutive terminal dilutions on Vero cells and then amplified three times in Vero cells.

Genetic characterization of recombinant chimeric PIV3-PIV2 viruses

The biologically-cloned chimeric PIV3-PIV2 viruses, rPIV3-2TM, rPIV3-2CT, rPIV3-2TMcp45, and rPIV3-2CTcp45, were propagated on LLC-MK2 cells and then concentrated. Viral RNAs extracted from pelleted viruses were used in RT-PCR amplification of specific gene segments using primer pairs specific to PIV2 or PIV3 (21, 22 or 23, 24 in Table 9). The restriction enzyme digestion patterns of the RT-PCR products amplified with PIV2 specific primer pairs from rPIV3-2TM, rPIV3-2CT, rPIV3-2TMcp45, and rPIV3-

2CTcp45, were each distinct from that derived from PIV2/V94, and their patterns, using EcoRI, MfeI, NcoI, or PpuMI, were those expected from the designed cDNA. Nucleotide sequences for the 8 different PIV3-PIV2 junctions in F and HN genes of rPIV3-2TM and rPIV3-2CT are given in Figure 9. Also, the *cp45* markers present in rPIV3-2TMcp45 and rPIV3-2CTcp45, except those in the 3'-leader region and the gene start of NP, were verified with RT-PCR and restriction enzyme digestion as previously described (Skiadopoulos et al., *J Virol.* 73:1374-81, 1999, incorporated herein by reference). These results confirmed the chimeric nature of the recovered PIV3-PIV2 viruses as well as the presence of the introduced *cp45* mutations.

PIV3-PIV2 recombinant chimeric viruses replicate efficiently in LLC-MK2 cells *in vitro*

The kinetics and magnitude of replication *in vitro* of the PIV3-PIV2 recombinant chimeric viruses were assessed by multicycle replication in LLC-MK2 cells (Figure 10). LLC-MK2 cell monolayer cultures in six-well plates were infected in triplicate with rPIV3-2TM, rPIV3-2CT, rPIV3-2TMcp45, or rPIV3-2CTcp45 at an MOI of 0.01 in the presence of p-trypsin (0.5 µg/ml). Samples were removed from culture supernate at 24 hour intervals for 6 days. Each of the recombinant chimeric viruses, except rPIV3-2CTcp45 (clone 2A1), replicated at the same rate and to a similar level as their PIV2/V94 parent virus indicating that PIV3-PIV2 chimerization of F and HN proteins did not alter the rates of growth of the recombinant chimeric viruses, and all reached a titer of 10⁷ TCID₅₀/ml or higher. Only the rPIV3-2CTcp45 grew slightly faster in each of two experiments and reached its peak titer earlier than PIV2/V94. This accelerated growth pattern was likely a result of an unidentified mutation in this clone since a sister clone failed to exhibit this growth pattern. rPIV3-2CTcp45 clone 2A1 was used in the studies described below.

The level of temperature sensitivity of rPIV3-2 chimeric viruses and their *cp45* derivatives

The level of temperature sensitivity of replication of PIV3-PIV2 recombinant chimeric viruses was tested to determine if rPIV3-2TM and rPIV3-2CT viruses exhibit a *ts* phenotype and to determine if the acquisition of the 12 *cp45* mutations by these viruses specified a level of temperature sensitivity characteristic of *cp45* derivatives bearing these 12 PIV3 *cp45* mutations (Skiadopoulos et al., *J Virol.* 73:1374-81, 1999, incorporated herein by reference). The level of temperature sensitivity of the virus was determined in LLC-MK2 cell monolayers as previously described (Skiadopoulos et al., *Vaccine* 18:503-510, 1999) (Table

13). The titer of rPIV3-2TM and rPIV3-2CT was fairly constant at permissive temperature (32°C) and the various restrictive temperatures tested indicating these recombinants were *ts*+. In contrast, their *cp45* derivatives, rPIV3-2TM*cp45* and rPIV3-2CT*cp45*, were *ts* and the level of temperature sensitivity was similar to that of rPIV3-1*cp45*, the chimeric PIV3-PIV1 virus carrying the complete PIV1 F and HN glycoproteins and the same set of 12 *cp45* mutations. Thus the *in vitro* properties of rPIV3-2TM and rPIV3-2CT viruses and their *cp45* derivative are similar to those of rPIV3-1 and rPIV3-1*cp45*, respectively, suggesting that the *in vivo* properties of the rPIV3-2 and rPIV3-1 viruses would also be similar, but surprisingly this was not the case.

Table 13. The replication of rPIV3-2CT and rPIV3-2TM are not temperature sensitive in LLC-MK2 cells, whereas the inclusion of the *cp45* mutations confers the *cp45* temperature sensitive phenotype

Titer at 32° C ^a		Change in titer (log ₁₀) at various temperatures compared to that at 32° ^{a, b}					
Virus	(log ₁₀ TCID ₅₀)	35° ^c	36°	37°	38°	39°	40°
rPIV3/JS	7.9	0.3 ^b	0.1	0.1	(0.3) ^b	(0.4)	0.4
PIV3 _{cp45} ^e	7.8	0.5	0.3	1.3	<u>3.4^d</u>	6.8	6.9
PIV1/Wash64 ^e	8.5	1.5	1.1	1.4	0.6	0.5	0.9
rPIV3-1	8.0	0.8	0.5	0.6	0.9	1.1	2.6
rPIV3-1 _{cp45}	8.0	0.5	0.4	<u>3.4^d</u>	4.8	6.6	7.5
PIV2/V9412 ^e	7.8	0.3	(0.1)	0.0	(0.4)	(0.4)	0.0
rPIV3-2CT	6.9	0.3	0.3	0.6	(0.1)	0.6	0.4
rPIV3-2TM	8.3	0.3	(0.1)	0.3	0.6	1.0	<u>2.1^d</u>
rPIV3-2CT _{cp45}	8.0	0.8	(0.4)	<u>2.0^d</u>	4.3	7.5	≥7.6
rPIV3-2TM _{cp45}	8.0	0.3	0.6	<u>2.4^d</u>	5.4	7.5	≥7.6

^a Data presented are means of two experiments.

^b Numbers not in parentheses represent titer decrease; numbers in parentheses represent titer increase.

^c Data at 35° were from one experiment only.

^d Values which are underlined represent the lowest temperature at which there was a 100-fold reduction of virus titer compared to the titer at permissive temperature (32°C). This restrictive temperature is referred to as the shut-off temperature.

^e Biologically-derived viruses.

rPIV3-2TM and rPIV3-2CT are attenuated, immunogenic, and highly protective in hamsters, and introduction of *cp45* mutations results in highly attenuated and less protective viruses

Hamsters in groups of six were inoculated intranasally with $10^{5.3}$ TCID₅₀ of rPIV3-2TM, rPIV3-2CT, rPIV3-2TM*cp45*, rPIV3-2CT*cp45*, or control viruses. It was previously seen that rPIV3-1 virus replicated in the upper and lower respiratory tract of hamsters like that of its PIV3 and PIV1 parents (Skiadopoulos et al., Vaccine 18:503-510, 1999; Tao et al., J. Virol. 72:2955-2961, 1998, each incorporated herein by reference). PIV2 virus replicates efficiently in hamsters, but rPIV3-2TM and rPIV3-2CT viruses each replicated to a 50- to 100-fold lower titer than their PIV2 and PIV3 parents in the upper respiratory tract and to a 320- to 2000-fold lower titer in the lower respiratory tract (Table 14). This indicates that the chimeric PIV3-PIV2 F and HN glycoproteins specify an unexpected attenuation phenotype in hamsters. rPIV3-2TM*cp45* and rPIV3-2CT*cp45*, the derivatives carrying the *cp45* mutations, were 50- to 100-fold more attenuated than their respective rPIV3-2 parents, with only barely detectable replication in the nasal turbinates, and none in the lungs. These rPIV3-2*cp45* viruses were clearly more attenuated than rPIV3-1*cp45*, exhibiting an additional 50-fold reduction of replication in the nasal turbinates. Thus, the attenuating effects of the chimerization of F and HN glycoproteins and that specified by *cp45* mutations were additive.

Table 14. The rPIV3-2TM and rPIV3-2CT viruses, in contrast to rPIV3-1, are attenuated in the respiratory tract of hamsters and importation of the *cp45* mutations resulted in further attenuation.

Virus ^a	Virus titers in the indicated tissue (log ₁₀ TCID ₅₀ /g ± S.E.) ^b [Duncan Group] ^c			
	NT	log ₁₀ titer reduction	Lung	log ₁₀ titer reduction
rPIV3/JS	5.9 ± 0.1[AB]	0	6.5 ± 0.1[A]	0
rPIV3 <i>cp45</i>	4.5 ± 0.2[C]	1.4 ^c	1.8 ± 0.2[E]	4.7 ^c
PIV1/Wash64 ^d	5.7 ± 0.1[B]	-	5.5 ± 0.1[B]	-
rPIV3-1	6.4 ± 0.2[A]	0	6.6 ± 0.2[A]	0
rPIV3-1 <i>cp45</i>	3.1 ± 0.1[D]	3.3 ^c	1.2 ± 0.0[F]	5.4 ^c
PIV2/V94 ^d	6.2 ± 0.2[A]	0	6.4 ± 0.2[A]	0
rPIV3-2CT	4.5 ± 0.4[C]	1.7 ^c	3.1 ± 0.1[D]	3.3 ^c
rPIV3-2TM	3.9 ± 0.3[C]	2.3 ^c	3.9 ± 0.4[C]	2.5 ^c
rPIV3-2CT <i>cp45</i>	1.4 ± 0.1[E]	4.8 ^c	1.5 ± 0.2[E]	4.9 ^c
rPIV3-2TM <i>cp45</i>	1.6 ± 0.2[E]	4.6 ^c	1.4 ± 0.1[E]	5.0 ^c

^a Hamsters in group of six were inoculated intranasally with 10^{5.5} TCID₅₀ of indicated virus on day 0.

^b Hamsters were sacrificed and their tissue samples harvested on day 4. The virus titer in hamster tissues was determined and the results are expressed as log₁₀TCID₅₀/g ± standard error (SE). NT = nasal turbinates.

^c The log₁₀ titer reduction values are derived by comparing: rPIV3*cp45* against rPIV3/JS; rPIV3-1*cp45* against rPIV3-1; each of the PIV3-PIV2 chimeras against PIV2/V94.

^d Biologically-derived viruses.

^e Grouping as analyzed by Duncan mult:range test.

To determine the immunogenicity and protective efficacy of the PIV3-PIV2 chimeric viruses, hamsters in groups of twelve were immunized with $10^{5.3}$ TCID₅₀ of rPIV3-2TM, rPIV3-2CT, rPIV3-2TM_{cp45}, rPIV3-2CT_{cp45}, or control viruses on day 0. Six of the hamsters from each group were challenged with 10^6 TCID₅₀ of PIV1 on day 29, and the remaining half were challenged with PIV2 on day 32. Hamsters were sacrificed 4 days after challenge and the lungs and nasal turbinates harvested. Serum samples were collected on day -3 and day 28, and their HAI antibody titer against PIV1, PIV2, and PIV3 was determined. As shown in Table 15, despite their attenuated growth in hamsters, immunization with rPIV3-2TM or rPIV3-2CT each elicited a level of serum HAI antibody against PIV2 that was comparable to that induced by infection with wild type PIV2/V94. Immunization of hamsters with rPIV3-2TM and rPIV3-2CT resulted in complete restriction of the replication of PIV2 challenge virus. rPIV3-2TM_{cp45} and rPIV3-2CT_{cp45} failed to elicit a detectable serum antibody response, and immunization of hamsters with either of these two viruses resulted in only a 10- to 100-fold reduction of replication of the PIV2 challenge virus in the lower respiratory tract (Table 15).

Table 15. The rPIV3-2CT and rPIV3-2TM viruses are highly protective in hamsters against challenge with wild type PIV2, but not against PIV1

Immunizing virus ^a	HA1 antibody titer ^b against indicated virus (reciprocal mean log ₂ ± SE)				Challenge virus titer ^c in indicated tissue (log ₁₀ TCID ₅₀ /g ± SE)			
	PIV1	PIV2	PIV3		PIV1		PIV2	
					NT	Lung	NT	Lung
rPIV3/JS	≤1	≤1	10.2±0.1		6.2 ± 0.2	5.8 ± 0.1	5.9±0.2	5.7±0.2
rPIV3 _{cp45}	≤1	≤1	8.6±0.2		5.9 ± 0.3	5.1 ± 0.3	5.6±0.2	4.5±0.7
PIV1	6.7±0.2	≤1	≤1		1.3 ± 0.1	≤1.2 ± 0.0	6.1±0.2	6.2±0.3
rPIV3-1	6.4±0.2	≤1	≤1		≤1.2 ± 0.0	≤1.2 ± 0.0	6.5±0.2	5.0±0.6
rPIV3-1 _{cp45}	1.8±0.6	≤1	≤1		3.9 ± 0.4	1.6 ± 0.3	6.2±0.2	4.5±0.6
PIV2	≤1	4.0±0.0	≤1		5.9 ± 0.2	5.5 ± 0.1	≤1.2±0.0	≤1.2±0.0
rPIV3-2CT	≤1	3.6±0.8	≤1		5.3 ± 0.1	5.2 ± 0.3	≤1.2±0.0	≤1.2±0.0
rPIV3-2TM	≤1	4.5±0.2	≤1		5.9 ± 0.2	4.4 ± 0.3	≤1.2±0.0	≤1.2±0.0
rPIV3-2CT. _{cp45}	≤1	≤1	≤1		6.2 ± 0.2	5.7 ± 0.1	5.3±0.2	3.3±0.8
rPIV3-2TM. _{cp45}	≤1	≤1	≤1		5.8 ± 0.3	4.4 ± 0.3	5.5±0.2	3.7±0.7

^a Hamsters in groups of 12 were immunized intranasally with 10^{5.3} TCID₅₀ of the indicated virus on day 0.

^b Serum samples were collected two days before immunization and 28 days after immunization. They were tested for HA1 antibody titer against the three PIVs, and the antibody titers are presented as reciprocal mean log₂ ± standard error (SE).

^c Six hamsters from each group were challenged intranasally with 10⁶ TCID₅₀ of PIV1 (on day 29) or PIV2 (on day 32). Hamster tissues were harvested 4 days after challenge, and the virus titer in indicated tissues are expressed as log₁₀TCID₅₀/g ± SE.

rPIV3-2TM and rPIV3-2CT are attenuated, immunogenic, and highly protective in AGMs, whereas introduction of *cp45* mutations results in highly attenuated and poorly protective viruses

Certain recombinant PIV3 and RSV viruses may exhibit different levels of attenuation in rodents and primates (Skiadopoulos et al., Virology In press, 1999; Whitehead et al., J Virol. 73:9773-9780, 1999, each incorporated herein by reference), indicating that attenuation can be somewhat species specific. Therefore, the rPIV3-2 viruses were evaluated for their level of replication and immunogenicity in AGMs. AGMs in groups of four were intranasally and intratracheally administered 10^5 TCID₅₀ per site of rPIV3-2TM, rPIV3-2CT, rPIV3-2TM*cp45*, rPIV3-2CT*cp45*, PIV2/V94, or rPIV3-1 on day 0. Virus in the NT swab specimens (collected day 1 to 12) and tracheal lavages (collected on day 2, 4, 5, 8, and 10) were titered as previously described (van Wyke Coelingh et al., Virology 143:569-582, 1985, incorporated herein by reference). As shown in Table 16, rPIV3-2TM and rPIV3-2CT were clearly attenuated in the respiratory tract of AGMs as indicated by a peak titer of virus shedding lower in both the upper and lower respiratory tract than their PIV2/V94 parent virus.

rPIV3-2TM*cp45* and rPIV3-2CT*cp45*, the derivatives carrying *cp45* mutations, were detected at very low levels, if at all, in the NT swab and tracheal lavage specimens suggesting that the attenuating effects of chimerization of the F and HN glycoproteins and that specified by the *cp45* mutations were additive for AGMs as well as for hamsters.

To determine whether immunization of AGMs with the PIV3-PIV2 chimeric viruses is protective against PIV2 challenge, AGMs previously infected with a rPIV3-2 virus were challenged with 10^5 TCID₅₀ of PIV2 on day 28 (Table 16). Virus present in the NT swab specimens (collected day 29 to 38) and tracheal lavages fluids (collected on day 30, 32, 34, 36, and 38) was titered as previously described (Durbin et al., Virology 261:319-30, 1999, incorporated herein by reference). As shown in Table 16, immunization with rPIV3-2TM and rPIV3-2CT induced a high level of restriction of the replication of PIV2/V94 challenge virus. In contrast, immunization of AGMs with rPIV3-2TM*cp45* and rPIV3-2CT*cp45* failed to restrict the replication of PIV2/V94 challenge virus and these animals developed very low levels of pre-challenge serum neutralizing antibody to PIV2. The complete restriction of replication of PIV2/V94 challenge virus in rPIV3-2CT immunized AGMs was associated with a 2.5-fold greater level of pre-challenge serum antibody to PIV2 than that of rPIV3-2TM immunized AGMs which provided incomplete protection.

Table 16. The rPIV3-2CT or rPIV3-2TM viruses are attenuated for replication in the respiratory tract of African green monkeys, yet still induce resistance to challenge with wild type PIV2

Immunizing ^a virus	Mean peak titer ^p of immunizing virus in indicated site (log ₁₀ TCID ₅₀ /ml ± SE)			Serum neutralization antibody titer ^c against indicated virus (mean reciprocal log ₂ ± SE)		Mean peak titer ^d of PIV2/V94 challenge virus in indicated site (log ₁₀ TCID ₅₀ /ml ± SE)	
	NT	TL		PIV1	PIV2	NT	TL
rPIV3-1	2.6±0.5	3.2±0.1		6.3±0.4	3.1±0.3	3.6±0.2	3.3±0.7
PIV2/V94	2.8±0.7	5.0±0.3		3.8±0.0	7.1±0.7	≤0.2	≤0.2
rPIV3-2CT	1.5±0.4	0.5±0.2		2.9±0.1	7.2±0.1	≤0.2	≤0.2
rPIV3-2TM	1.4±0.1	1.6±0.7		4.1±0.1	5.9±0.2	1.6±0.6	1.3±0.9
rPIV3-2CT _{cp45}	1.0±0.2	≤0.2		4.1±0.1	5.3±0.0	3.3±0.4	3.5±0.3
rPIV3-2TM _{cp45}	0.6±0.3	≤0.2		3.4±0.2	4.6±0.6	3.0±0.5	4.1±0.2

^a African green monkeys in group of 4 were inoculated with 10⁵ TCID₅₀ of indicated virus intranasally and intratracheally on day 0.

^b Combined nasal wash and throat swab (NT) samples were collected on days 1 to 12. Tracheal lavage (TL) samples were collected on days 2, 4, 6, 8, and 10. The virus titers were determined on LLC-MK2 monolayers and expressed as log₁₀TCID₅₀/ml ± standard error (SE).

^c Serum samples collected on day 28 were assayed for their neutralizing antibody titers against PIV1 and PIV2. The titers were expressed as reciprocal mean log₂ ± SE.

^d NT specimens were collected on days 29 to 38. TL specimens were collected on days 30, 32, 34, 36, and 38.

rPIV3-2TM is attenuated in its replication in the respiratory tract of chimpanzees

Chimpanzees in groups of 4 were inoculated intranasally and intratracheally with 10^5 TCID₅₀ of rPIV3-2TM or PIV2/V94 on day 0. NT swab specimens (day 1 to 12) and tracheal lavage (days 2, 4, 6, 8, and 10) samples were collected. Virus titer was determined as previously described (Durbin et al., Virology 261:319-30, 1999, incorporated herein by reference), and results are expressed as log₁₀TCID₅₀/ml. As shown in Table 17, rPIV3-2TM had a lower peak titer than its wild type parent PIV2/V94 and was shed for a significantly shorter duration than PIV2/94, indicating that rPIV3-2TM is attenuated in chimpanzees. PIV2/94 wt virus replicates to low levels in chimpanzees compared to hamsters and AFGs, whereas rPIV3-2TM virus was attenuated in each of these model hosts.

Table 17. rPIV3-2TM is attenuated in the respiratory tract of chimpanzees and yet still elicits a strong serum immune response to PIV2

Inoculated virus ^a	Mean peak titer ^b of virus shed in indicated site (\log_{10} TCID ₅₀ /ml \pm SE)		Mean days of virus shedding in the upper respiratory tract (days \pm SE)	Serum neutralizing antibody titer ^c against indicated virus (reciprocal mean $\log_2 \pm$ SE)	
	NT	TL		PRE	POST
PIV2/V94	2.9 \pm 0.6	1.2 \pm 0.5	8.8 \pm 1.1 ^d	$\leq 2.8 \pm 0.0$	6.2 \pm 0.5
rPIV3-2TM	2.0 \pm 0.3	$\leq 0.5 \pm 0.0$	2.5 \pm 0.7 ^d	3.3 \pm 0.2	4.3 \pm 0.4

^a Chimpanzees in group of four were inoculated intranasally and intratracheally with 10⁵ TCID₅₀ of indicated virus.

^b Nose/throat (NT) swab specimens and tracheal lavages (TL) were collected for 12 and 10 days, respectively, and virus titer were determined. The peak titers are expressed as \log_{10} TCID₅₀/ml \pm standard error (SE).

^c Serum samples collected 3 days prior and 28 days after virus inoculation were assayed for their neutralizing antibody titer against indicated virus. The titers are expressed as reciprocal mean $\log_2 \pm$ SE.

^dSignificant difference in duration of shedding, $p \leq 0.005$, Student T test.

Briefly summarizing the foregoing description and Examples, recombinant chimeric PIVs bearing heterologous viral genes or genome segments have been constructed in accordance with the description herein using a cDNA-based virus recovery system. Recombinant viruses made from cDNA replicate independently and can be propagated in the same manner as if they were biologically-derived viruses. In preferred embodiments, recombinant chimeric human PIV (HPIV) vaccine candidates bear one or more major antigenic determinant(s) of a HPIV, preferably in a background that is attenuated by one or more nucleotide modifications. Preferably, chimeric PIVs of the invention also express one or more protective antigens of another pathogen, for example a microbial pathogen. In these cases, the HPIV acts as an attenuated virus vector and is used with the dual purpose of inducing a protective immune response against one or more HPIVs as well as against the pathogen(s) from which the foreign protective antigen(s) was/were derived.

As mentioned above, the major protective antigens of PIVs are their HN and F glycoproteins. Thus, in exemplary embodiments, live attenuated PIV candidate vaccine viruses for use in infants and young children include chimeric HPIV3-1 and HPIV3-2 viruses carrying full-length PIV1 and partial PIV2 glycoproteins, respectively in a PIV3 background genome or antigenome. In the latter case, chimeric HN and F ORFs rather than full-length PIV2 ORFs are used to construct the full-length cDNA. The recovered viruses, designated rPIV3-2CT in which the PIV2 ectodomain and transmembrane domain is fused to the PIV3 cytoplasmic domain and rPIV3-2TM in which the PIV2 ectodomain was fused to the PIV3 transmembrane and cytoplasmic tail domain, possessed similar *in vitro* and *in vivo* phenotypes. In particular, the rPIV3-2 recombinant chimeric viruses exhibit a host range phenotype, i.e. they replicate efficiently *in vitro* but are restricted in replication *in vivo*. This attenuation *in vivo* occurs in the absence of any added mutations from *cp45*. This is an unexpected host range effect which is highly desirable for vaccine purposes, in part because the phenotype is not specified by point mutations which may refer to wt. At the same time, the unrestricted growth *in vitro* is highly advantageous for efficient vaccine production.

Although rPIV3-2CT and rPIV3-2TM replicate efficiently *in vitro*, they are highly attenuated in both the upper and the lower respiratory tract of hamsters and African green monkeys (AGMs), indicating that chimerization of the HN and F proteins of PIV2 and PIV3 itself specified an attenuation phenotype *in vivo*. Despite this attenuation, they are highly immunogenic and protective against challenge with PIV2 wild virus in both species. rPIV3-2CT and rPIV3-2TM were further modified by the introduction of the 12 PIV3 *cp45* mutations located outside of the HN

and F coding sequences to derive rPIV3-2CTcp45 and rPIV3-2TMcp45 which replicated efficiently *in vitro* but were even further attenuated in hamsters and AGMs indicating that the attenuation specified by the glycoprotein chimerization and by the cp45 mutations was additive.

The development of antigenic chimeric viruses possessing protective antigens of one virus and attenuating mutations from another virus has been reported by others for influenza viruses (Belshe et al., N. Engl. J. Med. 338:1405-1, 1998; Murphy et al., Infectious Diseases in Clinical Practice 2:174-181, 1993) and for rotaviruses (Perez-Schael et al., N. Engl. J. Med. 337:1181-7, 1997). Attenuated antigenic chimeric vaccines are more readily generated for these viruses which have segmented genomes, since genome segment reassortment occurs with high frequency during coinfection. Live attenuated influenza virus vaccine candidates are antigenically updated annually by replacement of the HA and NA genes of the attenuated donor virus with those of a new epidemic or pandemic virus. Recombinant DNA technology is also actively being used to construct live attenuated antigenic chimeric virus vaccines for flaviviruses and for paramyxoviruses. For flaviviruses, a live attenuated virus vaccine candidate for Japanese encephalitis virus (JEV) has been made by the replacement of the premembrane (prM) and envelope (E) regions of the attenuated yellow fever virus (YFV) with those from an attenuated strain of JEV (Guirakhoo et al., Virology 257:363-72, 1999). The JEV-YFV antigenic chimeric recombinant vaccine candidate was attenuated and immunogenic *in vivo* (Guirakhoo et al., Virology 257:363-72, 1999). Both the structural and the non-structural proteins of this chimeric virus came from a live attenuated vaccine virus. Antigenic chimeric vaccines have also been made between a naturally attenuated tick-borne flavivirus (Langat virus) and a wild type mosquito-borne dengue 4 virus, and the resulting recombinant was found to be significantly more attenuated for mice than its tick-borne parent virus (Pletnev et al., Proc. Natl. Acad. Sci. U S A. 95:1746-51, 1998), but this chimeric virus was highly restricted in replication in Vero cells *in vitro*. This is an example of an attenuating effect that stems from partial incompatibility between the evolutionarily divergent structural proteins specified by the Langat virus and the non-structural proteins of the dengue virus. A third strategy is being pursued for the production of a quadrivalent dengue virus vaccine in which a dengue 4 backbone containing an attenuating deletion mutation in the 3' non-coding region is used to construct antigenic chimeric viruses containing the protective antigens of dengue 1, 2 or 3 viruses (Bray et al., Proc. Natl. Acad. Sci. U S A 88:10342-6, 1991; J. Virol. 70:3930-7, 1996).

Antigenic chimeric viruses have also been produced for single-stranded, negative-sense RNA viruses. For example, antigenic chimeric PIV1 vaccine candidates can be constructed according to the methods disclosed herein by substituting the full-length HN and F proteins of parainfluenza virus type 1 for those of PIV3 in an attenuated PIV3 vaccine candidate, and this recombinant is attenuated and protective against PIV1 challenge in experimental animals. Similarly, exemplary antigenic chimeric respiratory syncytial virus (RSV) vaccine candidates can be made in which one or more of the RSV F and G protective antigens, or antigenic determinant(s) thereof, of subgroup B virus are substituted for those in an attenuated RSV subgroup A virus yielding attenuated RSV subgroup B vaccine candidates. (See also, International Publication No. WO 97/06270; Collins et al., Proc. Natl. Acad. Sci. USA 92:11563-11567 (1995); U.S. Patent Application No. 08/892,403, filed July 15, 1997 (corresponding to published International Application No. WO 98/02530 and priority U.S. Provisional Application Nos. 60/047,634, filed May 23, 1997, 60/046,141, filed May 9, 1997, and 60/021,773, filed July 15, 1996); U.S. Patent Application Serial No. 09/291,894, filed by Collins et al. on April 13, 1999; U.S. Provisional Patent Application Serial No. 60/129,006, filed April 13, 1999; U.S. Provisional Patent Application Serial No. 60/143,132, filed by Bucholz et al. on July 9, 1999; and Whitehead et al., J. Virol. 73:9773-9780, 1999, each incorporated herein by reference). When the glycoprotein exchanges between the PIV1 and PIV3 viruses and between the RSV subgroup A and RSV subgroup B viruses were performed in a wild type virus background, the antigenic chimeric viruses replicated to wild type virus levels *in vitro* and *in vivo*. These findings indicate that a high level of compatibility exists between recipient and donor viruses and that only very little, if any, attenuation was achieved as a result of the process of chimerization. These findings with the PIV1 and PIV3 and the RSV A and B glycoprotein exchanges contrast strikingly in several ways with those between PIV2 and PIV3 disclosed herein.

In the present disclosure, viable recombinant virus in which the full-length PIV2 HN or F protein was used to replace those of PIV3 was not recovered in this instance, evidently attributable to incidental mutations introduced during cDNA construction, whereas this was successfully achieved for the PIV1-PIV3 glycoprotein exchange. This suggests that the PIV2 HN or F glycoprotein is poorly compatible with one or more of the PIV3 proteins encoded in the cDNA. Two viable PIV2-PIV3 chimeric viruses were obtained when chimeric HN and F ORFs rather than full-length PIV2 ORF were used to construct the full-length cDNA. One of these chimeric viruses contained chimeric HN and F glycoproteins in which the PIV2 ectodomain was fused to the PIV3 transmembrane and cytoplasmic tail region, and the other contained chimeric HN

and F glycoproteins in which the PIV2 ectodomain and transmembrane region was fused to the PIV3 cytoplasmic tail region. Both rPIV3-2 recombinants possessed similar, although not identical, *in vitro* and *in vivo* phenotypes. Thus, it appeared that only the cytoplasmic tail of the HN or F glycoprotein of PIV3 was required for successful recovery of the PIV2-PIV3 chimeric viruses.

In previous studies directed to protein structure-function analysis, chimeric HN or F proteins have been constructed and expressed *in vitro* and have been used to map various functional domains of the proteins (Bousse et al., Virology 204:506-14, 1994; Deng et al., Arch. Virol. Suppl. 13:115-30, 1997; Deng, et al., Virology 253:43-54, 1999; Deng et al., Virology 209:457-69, 1995; Mebatsion et al., J. Virol. 69:1444-1451, 1995; Takimoto et al., J. Virol. 72:9747-54, 1998; Tanabayashi et al., J. Virol. 70:6112-6118, 1996; Tsurudome et al., J. Gen. Virol. 79:279-89, 1998; Tsurudome et al., Virology 213:190-203, 1995; Yao et al., J. Virol. 69:7045-53, 1995). In one report, a chimeric glycoprotein consisting of a measles virus F cytoplasmic tail fused to the transmembrane and ectodomains of the vesicular stomatitis virus G protein was inserted into a measles virus infectious clone in place of the measles virus F and HN virus glycoproteins (Spielhofer et al., J. Virol. 72:2150-9, 1998). A chimeric virus was obtained that was replication competent, but highly restricted in replication *in vitro* as indicated by delayed growth and by low virus yields indicating a high degree of attenuation *in vitro*. This finding is in marked contrast to the phenotype exhibited by recombinant PIV of the invention expressing chimeric glycoproteins, e.g., a PIV2-PIV3 chimera, which replicate efficiently *in vitro*.

The efficient replication of rPIV3-2 and other chimeric PIV viruses of the invention *in vitro* is an important property for a live attenuated vaccine candidate that is needed for large scale vaccine production. In contrast to rPIV3-2CT and rPIV3-2TM, rPIV3-1 was not attenuated *in vivo*. Thus, the chimerization of the HN and F proteins of PIV2 and PIV3 itself resulted in attenuation of replication *in vivo*, a novel finding for single-stranded, negative-sense RNA viruses. The mechanism for this host range restriction of replication *in vivo* is not known. Importantly, infection with these attenuated rPIV3-2CT and rPIV3-2TM vaccine candidates induced a high level of resistance to challenge with PIV2 indicating that the antigenic structure of the chimeric glycoproteins was largely or completely intact. Thus rPIV3-2CT and rPIV3-2TM function as live attenuated PIV2 candidate vaccine viruses, exhibiting a desirable balance between attenuation and immunogenicity in both AGMs and hamsters.

The attenuating effects of the PIV3-PIV2 chimerization of the F and HN glycoprotein are additive with that specified by the *cp45* mutations. rPIV3-2 recombinants containing the *cp45* mutations were highly attenuated *in vivo* and provided incomplete protection in hamsters against challenge with PIV2 and little protection in AGMs. This is in contrast to the finding with rPIV3-1*cp45* which was satisfactorily attenuated *in vivo* and protected animals against challenge with PIV1. The combination of the independent, additive attenuating effects of the chimerization of PIV3-PIV2 glycoproteins and the 12 *cp45* mutations appeared too attenuating *in vivo*. Clearly, if the rPIV3-2CT and rPIV3-2TM vaccine candidates are found to be insufficiently attenuated in humans, the *cp45* attenuating mutations should be added incrementally rather than as a set of 12 to achieve a desired balance between attenuation and immunogenicity needed for a live attenuated PIV2 vaccine for use in humans. The findings presented herein thus identify a novel means to attenuate a paramyxovirus and provide the basis for evaluation of these PIV3-PIV2 chimeric live attenuated PIV2 vaccine candidates in humans. Importantly, the rPIV3-2CT or rPIV3-2TM viruses can also be used as vectors for other PIV antigens or for other viral protective antigens, e.g., the measles virus HA protein or immunogenic portions thereof.

The present invention overcomes the difficulties inherent in prior approaches to vector based vaccine development and provides unique opportunities for immunization of infants during the first year of life against a variety of human pathogens. Previous studies in developing live-attenuated PIV vaccines indicate that, unexpectedly, rPIVs and their attenuated and chimeric derivatives have properties which make them uniquely suited among the nonsegmented negative strand RNA viruses as vectors to express foreign proteins as vaccines against a variety of human pathogens. The skilled artisan would not have predicted that the human PIVs, which tend to grow substantially less well than the model nonsegmented negative strand viruses and which typically have been underrepresented with regard to molecular studies, would prove to have characteristics which are highly favorable as vectors. It is also surprising that the intranasal route of administration of these vaccines has proven a very efficient means to stimulate a robust local and systemic immune response against both the vector and the expressed heterologous antigen. Furthermore, this route provides additional advantages for immunization against heterologous pathogens which infect the respiratory tract or elsewhere. These properties of PIV vectors are described herein above using examples of rPIV3 vectors which bear (i) a major neutralization antigen of measles virus expressed as a separate gene in wild type and attenuated backgrounds or (ii) major neutralization antigens of hPIV1 in place of the PIV3 neutralization antigens which express in addition a major neutralization antigen of HPIV2. These rPIV vectors were constructed

using wild type and attenuated backgrounds. In addition, the description herein demonstrates the ability to readily modify the level of attenuation of the PIV vector backbone. According to one of these methods, varying the length of genome inserts in a chimeric PIV of the invention allows for adjustment of the attenuation phenotype, an effect which was pronounced in attenuated viruses both
5 which is only apparent in derivatives of wild type viruses using very long inserts.

Although the foregoing invention has been described in detail by way of example for purposes of clarity of understanding, it will be apparent to the artisan that certain changes and modifications may be practiced within the scope of the appended claims which are presented by way of illustration not limitation. In this context, various publications and other references have
10 been cited within the foregoing disclosure for economy of description. Each of these references are incorporated herein by reference in its entirety for all purposes.